REMARKS

Claims 1-17 were pending in this application. Claims 1 and 2 are cancelled herein, and claims 3-17 are amended herein. Entry of these amendments is respectfully requested. Upon entry of these amendments, claims 3-17 remain pending in this application.

On May 10, 2004, a telephonic interview was conducted between the undersigned Attorney for Applicant and Examiner Vogel. During that interview Attorney for Applicant presented technological bases directed to overcome the obviousness rejection based on Sauer (US Pat. No. 4,959,317) and Berkner (Curr. Top. Microbiol. Immunol., 158:39-66, 1992) references. More specifically, Attorney for Applicant discussed differences between herpesviruses and adenoviruses, concluding that, at the time of Applicant's invention, there was not a reasonable expectation of success in applying to adenoviruses the methods in the Sauer '317 patent. No resolution of the issues was achieved, and at the end of the interview it was agreed that Applicant would provide evidence of the technological bases leading to this conclusion, and that the Examiner would consider this evidence.

As part of this response Dr. Frank L. Graham, a co-inventor and expert in the field of recombinant virus technologies, provides a declaration under 37 CFR 1.132. References that are discussed in this declaration also are included, and comprise part of the evidence requested by the Examiner. Entry and consideration of Dr. Graham's Declaration and the associated references is respectfully requested.

Drawings

As discussed in the last Response (mailed 12/10/2003 and received at the Patent Office 12/16/2003), Figure 6B was inadvertently omitted in the filing of this application, but this figure is present in application serial number 08/486,549, filed 6/7/1995, to which priority is claimed. Applicant appreciates that this inadvertent error creates a problem for the USPTO as far as its

amendment practice. In that Figure 6B is not essential to the enablement or other requirements of the present invention, the specification has been amended so that references to Figure 6B are removed. It is Applicant's understanding that Figure 6B currently is not incorporated in the specification (page 2 of 09/10/2003 Office action), so no amendment to the drawings appears necessary to remove Figure 6B from the specification at this time.

Sequence Compliance

The Examiner, in the 03/09/2004 Final Office action, stated that the CRF in the most recent submission was found to be unreadable. Accordingly, in order to comply with this deficiency, Applicant provides herewith a replacement CRF, a replacement paper copy, and the following statements regarding these. A copy of the CRF Problem Report also is provided.

The following statements are provided with regard to the provided Sequence Listing.
I hereby state that the substitute/replacement copy of the computer readable form, submitted in accordance with 37 CFR 1.825(d), is identical to that originally filed.
Further, the Applicant states that the paper copy of the "Sequence Listing" in the present application is identical to the computer readable format copy enclosed herewith. I hereby state that the substitute/replacement copy of the computer readable form and the paper copy of the Sequence Listing do not include new matter.

Should the U.S. Patent Office find that any requirement has not been fully and properly met, it is respectfully requested that the Attorney indicated below be contacted by telephone and provided an opportunity to fully comply with all requirements under 37 C.F.R. 1.821-1.825.

Specification

To comply with the objection under 35 USC 132, Applicant has removed by amendment herein the sentence "All applications for which priority is claimed are hereby incorporated by

reference." Applicant notes that priority to these applications remains in force. Applicant also has removed by amendment the two references to Figure 6B in the specification.

Also in the amendments to the specification herein, Applicant requests entry of the REPLACEMENT CRF sequence listing and the identical paper copy, both enclosed herewith. Because the REPLACEMENT CRF is on a 3.5-inch floppy diskette, the paragraph describing the form of the CRF also is amended herein

Claim Rejections - 35 USC § 112, second paragraph

Claims 1-17 stand rejected under 35 USC §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention.

Per discussion during the informal interview with the Examiner, claims 1 and 2 are cancelled, and amendments to other claims are directed toward overcoming this basis for rejection. Exemplary of such amendments is the amended portion of claim 3, ". . . inserted expression cassette comprising a promoter sequence, a coding sequence of a gene, and a transcription termination site, and site-specific recombinase target sites positioned to remove or invert a portion of the expression cassette ~~operably linked to the gene~~, . . . ". The claim as amended now specifies which basic regulatory elements are present in the cassette. Support for the inclusion of these cassette elements is found, *inter alia*, on page 12, lines 20-25, on page 13, lines 17-20, and page 16, lines 9-20. In that various rearrangements may be employed to control gene expression of a gene in the cassette, as disclosed in the specification, the phrase "positioned to remove or invert a portion of the expression cassette" replaces "operably linked to the gene." Support for this part of the amendment is found, *inter alia*, on page 22, line 14 to page 23, line 6 (see last sentence), page 26, line 7 to page 27, line 22 (with inversion discussed on page 26, line 26). Also, support for amendments to claims 14 and 16 is found, *inter alia*, on page 27, line 15 to 22, and page 28, line 20 to page 29, line 1.

Dependent claims 5, 7, 9, 11, 13, 15 and 17 have been amended only by insertion of — coding sequence of the - - before "gene" to comply with the Examiner's first basis for rejection under 35 USC §112, second paragraph rejection (see page 4 of 09/10/2003 Office action, and page 4 of 03/09/2004 Final Office action) in view of the previous and current amendments to their respective independent claims. As stated for previous similar amendments, these are believed to clarify, and not narrow, the scope of the claims. The same applies to other amendments made herein to conform to the Examiner's concerns expressed with regard to 35 USC §112, second paragraph. Also, these amendments do not add new matter.

Also, Applicant respectfully makes the following point regarding the statement made in the 03/09/2004 Final Office action on page 4, "Furthermore, if one assumes that the term "expression cassette" includes a promoter for expression of the recited gene, then expression would take place regardless of the control of the recited site-specific recombinase." To assure that this is not taken out of context at a later date by a third party, Applicant points out that this quoted statement would not hold true, at a minimum, for an embodiment in which there is a spacer inserted between the promoter and the coding sequences for the gene of the expression cassette. In such configuration one would not expect expression of the gene until a recombinase is introduced and the recombinase action removes the spacer (see Example 5, particularly page 21, lines 14-25).

Claim Rejections - 35 USC § 103

Claims 1-17 stand rejected under 35 USC § 103(a) as being unpatentable over Sauer (US Patent No.: 4,959,317) in view of Berkner (Curr. Top. Microbiol. Immunol., 158:39-66, 1992). As is well recognized, a *prima facie* case for obviousness requires 1) some suggestion or motivation to modify a single reference or combine the teachings of two or more references, 2) a reasonable expectation of success, and 3) a teaching or suggestion of all claim elements by the prior art reference(s). (MPEP 2143)

During the informal telephonic interview of May 10, 2004, Attorney for Applicant provided a number of technological bases for the conclusion that, given the differences between pseudorabies virus (a herpes virus) utilized by Sauer and adenoviruses, there was not a reasonable expectation of success to achieve in adenoviruses what has been arguably disclosed to have worked in Sauer with recombinases in pseudorabies vectors. Attorney for Applicant discussed certain references that provided evidence of these differences.

During that informal interview, the Examiner requested that evidence be provided to substantiate certain points made. Toward this end, evidence is provided in the form of Dr. Graham's Declaration and the articles referenced therein, as well as in discussion of such references (in #2 below). Evidence and arguments also are provided that dispute that the requirements for the first element of the *prima facie* case for obviousness have been met (in #1 below).

The arguments and evidence are subdivided into two general issues: 1) It is improper to interpret the paragraph in Sauer's '317 patent, col. 4, starting on line 27, to indicate that that "any virus could be used in the place of pseudorabies virus" when this is not explicitly stated and, in the alternative, without proper regard for differences and difficulties expected among different viruses, and 2) A number of DNA replication differences exist between herpes viruses and adenoviruses, and in view of one or more of these differences, it was not reasonable for one skilled in the art at the time of the present invention to expect success for the use of recombinases and recombinase target sites in adenoviruses. These are discussed in the sections below.

<u>1) It is improper to interpret the paragraph in Sauer's '317 patent, col. 4, starting on line 27, to indicate that that "any virus could be used in the place of pseudorabies virus" when this is not explicitly stated and, in the alternative, without proper regard for differences and difficulties expected among different viruses.</u>

Near the beginning of the Detailed Description of the Invention of the '317 patent, Sauer et al. provide a general paragraph, starting on line 27 of col. 4, indicating that a DNA sequence may be introduced into eukaryotic cells by methods known in the art. Included in that paragraph is the sentence, "As used herein, the term "vector" includes plasmids and viruses." In contrast to what is actually stated in this paragraph, the 09/10/2003 Office action states, "The reference discloses that any vector may be used, including viruses (column 4, lines 33-35)" (page 8, underline emphasis added). Further, the 03/09/2004 Final Office action stated this paragraph to indicate that "any virus could be used in the place of pseudorabies virus" (page 5, underline emphasis added). However, Applicant respectfully asserts that the insertion of "any" into the above-quoted sentence in the noted paragraph is unwarranted broadening that takes the sentence and the paragraph out of context. This insertion is without evidentiary support on the part of the Patent Office, and therefore is not proper. Accordingly, and quite simply, under any reasonable reading of the paragraph, no basis exists to add "any." Thus, the Patent Office has erred in its additions of the word "any" in its Office actions, and this has contributed to an erroneous use of the '317 patent in the obviousness rejection. This factual conclusion counters the first element of the Patent Office's *prima facie* case for obviousness because Sauer's '317 patent does not provide a suggestion or a motivation to be combined with adenovirus references such as Berkner.

Additionally, and in the alternative, Applicant provides evidence to indicate there was a recognition on the part of Dr. Sauer, a co-inventor of the '317 patent and co-author of a scientific article referenced below, that the methods used in pseudorabies in the '317 patent were not broadly applicable to all viruses. This evidence supports a clearly limited interpretation of how far the method was perceived to be applicable, and thus the true meaning of the referenced paragraph (both technically and legally). As such, it directly opposes any conclusion that there would have been a reasonable expectation of success applying to adenoviruses what Sauer et al. demonstrated in pseudorabies in the '317 patent.

This evidence is found in the concluding sentences in Gage, Sauer, Levine and Glorioso, "A Cell-free recombination system for the site-specific integration of multigenic shuttle plasmids

into the herpes simplex virus type 1 genome," J. Virol 66: 5509-5515, 1992 (copy attached to Declaration of Dr. Graham). There, after discussing three other advantages of the method, the fourth advantage is stated "Finally, the Cre-lox-based recombination system should be useful for viral systems for which marker transfer procedures are currently difficult or unavailable. These include cytomegaloviruses, Epstein-Barr virus, and varicella-zoster virus." (page 5514) As noted in Dr. Graham's Declaration, with these final two sentences the authors of this paper, which include Sauer, the lead named inventor on the '317 patent, are stating only that viruses from the herpes family are likely to benefit from this method. Based on this evidence, it is most reasonable to conclude that the inventors' understanding of the paragraph starting on line 27 of col. 4 of the '317 patent did not mean that there was a reasonable chance of success that any virus could be adapted to work as successfully and predictably as the Cre-lox-based recombination system was believed would be useful for herpes viruses. This is supported by the three other advantages stated in the last paragraph on page 5514, two pertaining to superiority over marker transfers (which the above quotation indicates are a difficulty for the named herpes-family-member viruses), and the third pertaining to evidence that recombination efficiencies for Herpes Simplex Virus (HSV) were even better than for the previously reported pseudorabies virus (thus still focusing exclusively on herpes viruses). Clearly the focus is on herpes viruses, and not on all or any viruses.

Further, in view of the Gage et al. reference, a proper interpretation of the sentence in '317 starting on line 32 of col. 4, "As used herein, the term "vector" includes plasmids and viruses" is that whether the pseudorabies or related DNA construct was in the form of a plasmid or a functional virus, such construct is still considered a vector. Thus, this sentence is meant to ensure that both plasmid and virus forms are considered to be "vectors" in the '317 specification and claims (and not that all plasmids and all viruses are considered suitable vectors).

Another argument pertains to the fact that there is more than one interpretation for the paragraph, starting on line 27 of col. 4, of the '317 patent. Namely, later in that patent it is stated that in one embodiment the methods disclosed involve two or more introductions of a DNA sequence into a

eukaryotic cell (see col. 5, lines 30-50). One or two of such introductions provide a DNA sequence that has recombinase target sites placed in a specific location relative to functional parts of the DNA sequence (see col. 5, lines 53-68). Another introduction involves adding Cre DNA, preferably under control of a regulatory nucleotide sequence (see col. 5, lines 51-53). While the first phrase "introducing a DNA sequence" of the paragraph, starting on line 27 of col. 4, and its later sentence, "As used herein, the term "vector" includes plasmids and viruses," might be read broadly to include all such DNA introductions, this paragraph also can be interpreted to refer only to the DNA introductions that introduce DNA into the cell that is comprised of recombinase target sites (i.e., the DNA sequences that are to be manipulated by Cre). Even if the paragraph were interpreted to refer to all DNA introductions, this nonetheless does not *de facto* mean that Sauer et al. considered that any virus, or that all viruses, would readily work well in place of the viruses they had developed to introduce DNA for either or all introductions. Thus, this multiple interpretation supports, at a basic level, doubt as to the applicability of this paragraph's disclosure to the Patent Office's obviousness rejection.

Accordingly, the above provides evidence against two of the elements of the prima facie case of obviousness. First a proper interpretation of the paragraph starting on line 27 of col. 4 of the '317 patent does not provide a suggestion or motivation to combine the '317 teachings with an adenovirus reference such as Berkner. This is because the referenced paragraph does not broadly refer to all (i.e., <u>any</u>) viruses, and, alternatively, does not refer to both DNA introductions. Further, the quotation from the Gage et al. article provides direct evidence that the lead inventor of the '317 patent did not perceive applicability for the '317 method beyond the herpes viruses. Alone or in combination with the evidence in the next section (and the Declaration of Dr. Graham), this provides evidence that there was no reasonable chance of success of using the methods disclosed in the '317 patent other than with herpes viruses. Based on the above, reconsideration and withdrawal of the obviousness rejection is respectfully requested.

<u>2) DNA replication differences exist between herpes viruses and adenoviruses, and in view of one or more of these differences, it was not reasonable for one skilled in the art at the time of the</u>

present invention to expect success for the use of recombinases and recombinase target sites in adenoviruses.

Absolute predictability is not required to support an obviousness rejection, but at least some degree of predictability is required (MPEP 2143.02). Applicant respectfully asserts that, in view of one or more pieces of evidence showing differences between pseudorabies/herpes viruses and adenoviruses, and unpredictability in certain aspects of adenovirus replication and recombination, summarized below and discussed in the attached Declaration of Dr. Frank L. Graham, the degree of predictability is insufficient for applying Sauer's teachings of recombinase and recombinase target sites to adenoviruses. Accordingly, based on this evidence, the *prima facie* case for obviousness has not been met and should be withdrawn.

Key differences in the replication machinery and methods of pseudorabies/herpes viruses and adenoviruses are summarized below, as are aspects of unpredictability with regard to adenovirus replication and recombination. Detailed explanation based on attached scientific references is found in the attached Declaration of Dr. Frank L. Graham, full consideration of which (including the attached references) is requested during reconsideration of the obviousness basis for rejection. (To save time and space, the points are summarized below, rather than re-presented from the Declaration.)

The key differences and aspects of unpredictability:

1. Herpes viruses encode most of their own replication enzymes. Adenovirus DNA replication uses a unique mechanism, using its own DNA polymerase as well as cell enzymes, and involving long stretches of single-stranded DNA as intermediates in the DNA replication. The long stretches of single stranded DNA introduce uncertainty as to whether partially palindromic sequences, such as the loxP target sites of Cre, would be tolerated by the adenovirus replication machinery. Also working against a reasonable expectation of success were the higher possibilities of self annealing or hair pin formation that could interfere with the Ad DNA polymerase.

2. Recombination, which is much more prevalent in adenoviruses than in herpes viruses, adds to the unpredictability of the outcome with adenoviruses, and, consequently, there is an insufficient expectation of success when applying the methods shown to work in pseudorabies to adenoviruses.

3. Splicing of mRNAs, to yield different proteins from a given span of DNA, is much more prevalent in adenoviruses than in herpes viruses. This increases the unpredictability of the outcome with adenoviruses. Consequently, there is an insufficient expectation of success when applying the methods shown to work in pseudorabies to adenoviruses.

In conclusion, reconsideration and withdrawal of the obviousness rejection for remaining claims 3-17 is respectfully requested. This is requested in view of the substantial evidence provided herein, including the Declaration of Dr. Graham and its attached references. It is believed that based on the weight of the evidence provided herein, in comparison to the evidence for the case of obviousness, the obviousness rejection is improper and should be withdrawn.

* * * * * * *

All claims having been placed in condition for allowance, expedited passage of this case to issuance is respectfully solicited.

Applicant requests that the Examiner call the undersigned if clarification is needed on any aspect of this response, or if the Examiner believes that any valid basis of non-patentability

remains after entrance and consideration of the remarks and amendments presented herein.

Respectfully submitted,

5/24/04

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DECLARATION OF FRANK GRAHAM; PhD.
Examining Group 1636
Patent Application
Docket No. AdVec10IA-C5A (new docket no.: 10524-003)
Serial No. 09/981,685

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:		Frank L. Graham. et al.
Examiner	:	Vogel, Nancy T.
Art Unit	:	1636
Docket No.	:	Docket No. AdVec10IA-C5A (new docket no.: 10524-003)
Serial No.	:	09/981,685
Filed	:	10/17/2001
For:		ADENOVIRUSES FOR CONTROL OF GENE EXPRESSION

Commissioner of Patents
P.O. Box 1450
Alexandria, VA, 22313-1450

Dear Sir or Madam:

DECLARATION OF FRANK L. GRAHAM, Ph.D., UNDER 37 CFR 1.132

I, Frank L. Graham, Ph.D., hereby declare and say as follows:

THAT I am currently a Visiting Professor at the Istituto di Richerche di Biologia Molecolare P. Angeletti Via Pontina Km. 30.600, 00040 Pomezia (Roma) Italy;

THAT I was formerly employed. for a period of 27 years, as Professor in the Department of Biology and the Department of Pathology, McMaster University, Hamilton, Ontario;

THAT I earned my Ph.D. in the Department of Medical Biophysics, at the University of Toronto, Toronto, Ontario, Canada in 1970;

THAT I am one of the above-named Applicants and inventors of the subject matter

described and claimed in the above-identified patent application;

THAT, I have studied the application Serial No. 09/981,685 and all office actions which have been issued during prosecution of this application, as well as all responses which have been filed on the Applicants behalf. I further understand that two of the issues in the obviousness rejection in view of Sauer and Berkner references (discussed in the 09/10/2003 and 03/09/2004 Office actions) pertain to 1) the meaning of the paragraph beginning on column 4, line 27 of Sauer's US 4,959,317 and 2) the reasonable expectation of success of applying the techniques used by Sauer with pseudorabies-based vectors to adenoviruses. As to these issues, and being thus duly qualified, I declare as follows:

1) I hereby declare that with respect to the paragraph beginning on column 4, line 27 of Sauer's US 4,959,317 which states "Methods for introducing a DNA sequence into eukaryotic cells are known in the art. These methods typically include the use of a DNA vector to introduce the sequence into the DNA of a single or limited number of eukaryotic cells and then growing such cell or cells to generate a suitable population of cells. As used herein, the term "vector" includes plasmids and viruses. Preferably, the DNA sequences are introduced by a plasmid capable of transforming a selected eukaryotic cell while carrying a DNA sequence. The particular vector which is employed to introduce the DNA sequence into a selected eukaryotic cell is not critical. In a preferred embodiment, DNA sequences are introduced into mammalian cells according to the CaPO.sub.4 transfer procedure described by Graham and van den Eb, Virology, 52:456-467 (1973)." that the examiner's interpretation of the expression "the term "vector" includes plasmids and viruses" to mean that "any virus could be used" in the practice of the '317 invention is incorrect for the following reasons:

1. In the quoted paragraph Sauer is clearly discussing methods for establishing cell lines that have incorporated a DNA sequence carried by the DNA vector. This is the standard meaning of the expression "transforming a selected eukaryotic cell". In addition, in the sentence beginning "These methods typically include....." it is clear that Sauer is referring to transformation of eukaryotic cells since after introducing "the sequence into the DNA of a single .....cell(s)" he

says "then growing such cell or cells to generate a suitable population of cells" which is what one typically does to establish a population of transformed cells. Sauer goes on to say "In a preferred embodiment, DNA sequences are introduced into mammalian cells according to the CaPO.sub.4 transfer procedure described by Graham and van den Eb, Virology, 52:456-467 (1973)." At the time the '317 application was filed, the CaPO4 technique of Graham and Van der Eb was the standard technique for transformation of mammalian cells and was widely used for exactly this purpose.

Furthermore, in the context of the quoted paragraph it would be incorrect to state that "any virus could be used" to transform cells and it is noteworthy that Sauer does not state this. For example, a lytic virus such as a Pox virus would not be a suitable vector for transformation of a eukaryotic cell because the host cell would be killed by the virus and it would be impossible to subsequently grow "such cell or cells to generate a suitable population of cells".

2) I herby declare that the methods for construction and use of adenovirus vectors that were known in the art at the time the captioned application was filed, combined with the disclosure made by Sauer in US 4,959,317 on the use of the Cre-lox system in pseudorabies based vectors, were insufficient to provide a reasonable expectation of success in applying these same methods in adenovirus based vectors for the following reasons:

1. Pseudorabies is a member of the Herpes Virus family of viruses which is a distinct class of DNA viruses from the Adenoviridae. Herpes viruses and adenoviruses differ in many aspects that could have affected our ability to insert within an adenovirus vector a cassette of the kind taught in the captioned application, and to achieve success in the consistent expression of gene(s) in that cassette. For example as stated in Challberg and Kelly's article pertaining to adenoviruses, Ann. Rev. Biochem. 58: 671-717, 1989., on page 674 "Thus, the products of the first stage of DNA replication are a daughter duplex and a displaced single strand." And further in the same paragraph "Following a second initiation event, complementary strand synthesis proceeds from one end of the template to the other, generating a second daughter duplex. In both stages of

adenovirus DNA replication there is only one priming event per nascent daughter strand, so all viral strands are synthesized in a continuous fashion from their 5' termini to their 3' termini. And at the bottom of page 674 Challberg and Kelly state: "Initiation of adenovirus DNA replication occurs by a novel mechanism in which the first nucleotide in the new DNA chain becomes covalently linked to a virus-encoded protein, the terminal protein precursor. This mechanism is unique among the DNA viruses of mammals...." Furthermore Roizman in Virology, Second Edition, B.N. Fields Ed. Chapter 5 Multiplication of Viruses, pp 87-94. 1990. states on page 93 left column middle: "Adenoviruses encode a DNA polymerase but depend on the host cells for many of the other functions involved in the synthesis of their DNA. At the other extreme are the herpesviruses. Herpes simplex viruses encode numerous proteins involved in the pathway of synthesis of DNA." Roizman further states in Chapter 65 of the same volume, on page 1807 " A characteristic of herpesviruses not shared by other nuclear DNA viruses is that they specify a large number of enzymes involved in DNA synthesis."

Thus Adenovirus DNA replicates by a unique mechanism, one that is distinct from that employed by Herpes viruses, and utilizing different polymerases and other DNA replicative machinery. In particular the replication of the Ad genome involves a process that utilizes extensive single stranded DNA templates as intermediates in the DNA replication process. Because of the involvement of long stretches of single stranded DNA in the replication of Ad DNA, there was uncertainty as to whether partially palindromic sequences as represented by the loxP target sites of Cre, and more particularly multiple repeats of such sequences would be tolerated by the Ad replication machinery since there could be a greater probability of self annealing or hair pin formation under such circumstances that could interfere with the Ad DNA polymerase. Accordingly, what was shown to work in a herpes virus which has its own specific replication enzymes, does not provide evidence that there was a reasonable chance of success in a different virus class, the Adenoviridae, which do not share those enzymes and which in fact have their own specific DNA replication mechanism. It is noted that this evidence as to there not being a reasonable expectation of success, should be considered on its own and, alternatively, in combination with the other factors provided below.

2. As stated by Young and Silverstein The kinetics of adenovirus recombination in homotypic and heterotypic genetic crosses, Virology 101, 503-515, 1980 and as was well known in the art, "Adenoviruses undergo extensive recombination during the course of a productive mixed infection in cultured animal cells". It could not have been predicted that a sequence bounded by directly repeated loxP sites might not be excised by spontaneous recombination at such a high frequency that the methods taught in the captioned application might not be operable in practice. Indeed, as stated in the captioned application (page 20, line 27 to page 21, line 12, and Figure 5C, comparing two most right columns of both graphs) spontaneous recombination was determined to occur at detectable levels (estimated from measurement of luciferase levels to be about 1% of the levels seen when Cre was present to induce site specific recombination). This recombination could not have been predicted to have been at this tolerable level; it could have been substantially higher and could have interfered with the method. In marked contrast, Sauer saw no evidence for spontaneous recombination in the pseudorabies virus system (see Tables 4-1 and 4-2 showing that in the absence of Cre PRV42::pBS64 produced no black plaques that would result from excision of pBS64. The efficiency of spontaneous recombination from the data presented by Sauer was less than about 0.01% or at least 100 times lower than in the Ad system). Thus there was a clear difference between the spontaneous recombination frequency in the Ad vector system compared to pseudorabies although the methods taught in the captioned application were ultimately determined useful, in spite of the level of recombination, upon conducting the experiments in the captioned application.

3. During adenovirus replication there is extensive processing of viral RNA transcripts to mRNAs, and in particular most mRNAs are generated by RNA splicing of larger transcripts. (Indeed RNA splicing was first discovered in the adenovirus system.) This is true of most E1 transcripts as it is for transcripts from other regions of the Ad genome. In contrast, as pointed out by Roizman, in Chapter 65 of the above cited volume, on page 1814 "Notwithstanding the efficient expression of HSV genes in the environment of higher eukaryotic cells, only a relatively small proportion of HSV mRNAs are derived by splicing." Indeed, in work done in the

Declarant's laboratory in 1988 (Johnson et al., Abundant expression of herpes simplex virus glycoprotein gB using an adenovirus vector, Virology 164: 1-14, 1988) when a cassette containing an SV40 promoter was placed in E3 of an Ad vector it was found that expression of the inserted gB DNA sequences was dependent not on mRNAs regulated by the SV40 promoter element but by splicing from RNA transcripts generated from a promoter or promoters upstream. On page 11 of the cited article is stated: "From the size and heterogeneity of cDNA species produced by primer extension it seems certain that at least some of the transcripts must initiate in upstream Ad5 sequences, and possibly involve the use of more than a single promoter and a variety of different splicing events." Thus in the Ad system the consequences of RNA splicing in terms of their effects on expression of inserted foreign DNA are not always predictable. Consequently, it could not have been predicted whether insertion of a more or less randomly chosen spacer DNA between a promoter and a cDNA in an Ad vector might have failed to prevent expression of the downstream cDNA coding sequences if, for example, a splicing event utilizing cryptic splice sites that might be present in the spacer, effectively removed the spacer segment from the transcript.

4. To the best of my knowledge no published reports had documented the insertion into adenovirus vectors of partially palindromic sequences such as those represented by lox sites either as single inserts or as multiple inserts at the time the captioned application was filed. Indeed, in the article by Gage, Sauer, Levine and Glorioso A Cell-free recombination system for the site-specific integration of multigenic shuttle plasmids into the herpes simplex virus type 1 genome. J. Virol 66: 5509-5515, 1992 it is stated "Finally, the Cre-lox-based recombination system should be useful for viral systems for which marker transfer procedures are currently difficult or unavailable. These include cytomegaloviruses, Epstein-Barr virus, and varicella-zoster virus." Since Sauer is inventor on the US 4,959,317 patent, used in the obviousness rejection, it is noteworthy that these authors suggest the use only of viruses from the herpes family. This provides evidence of the limited applicability of the method, as far as virus classes, as perceived by a group of scientists that include Sauer. Further, this statement, which must be attributed to Sauer as a co-author, diminishes the scope that should be attributed to the paragraph

beginning on line 27 of col. 4 of the Sauer US 4,959,317 patent. That is, this statement provides evidence against the statement by the Examiner that Sauer indicated that ". . . any virus could be used in place of pseudorabies virus . . .".

Copies of the articles cited above are provided as an attachment to this declaration.

Accordingly, in my opinion, and supported by the evidence of the articles attached to this declaration and explained above, given the very great differences between herpes viruses and adenoviruses in their mechanisms of DNA replication and degree of RNA splicing and the documented difference between the results of Sauer in studies of a pseudorabies vector versus our results with an Ad vector in terms of spontaneous recombination of DNA flanked by loxP sites, and given the lack of any teaching in the art showing that DNA segments flanked by lox P sites could be cloned in Ad vectors in such a way as to functionally regulate expression of a downstream of a coding sequence, the obviousness rejection that has been advanced in the present application should be reconsidered by the US Patent Office and withdrawn.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements in the like so made are punishable by fine or imprisonment, or both, under section 1001 of title 18 of the U.S.C. and that such willful false statements made jeopardize the validity of the application or of any patent issuing thereon.

Further declarant sayeth naught.

F. L. Graham

Frank Graham, Ph.D.

May 20, 2004

Date

1600

BIOTECHNOLOGY SYSTEMS BRANCH

CRF Problem Report

The Scientific and Technical Information Center (STIC) experienced a problem when processing the following computer readable form (CRF):

Application Serial Number: 09/981,685
Filing Date: 10/17/01
Date Processed by STIC: 12/23/03

STIC Contact: **Mark Spencer: Telephone: 703-308-4212; Fax: 703-308-4221**
***Effective 12/13/03*: Telephone: 571-272-2510; Fax: 571-273-0221**

Nature of Problem:

The CRF (was):
- ✓ (circle one) Damaged or Unreadable (for Unreadable, see attached)
- ____ Blank (no files on CRF) (see attached)
- ____ Empty file (filename present, but no bytes in file) (see attached)
- ____ Virus-infected. Virus name: _____ The STIC will not process the CRF.
- ____ Not saved in ASCII text
- ____ Sequence Listing was embedded in the file. According to Sequence Rules, submitted file should **only** be the Sequence Listing.
- ____ Did not contain a Sequence Listing. (see attached sample)
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A Cell-Free Recombination System for Site-Specific Integration of Multigenic Shuttle Plasmids into the Herpes Simplex Virus Type 1 Genome

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This report describes a novel method for complementation studies of defective herpes simplex virus (HSV) genes. Viral test gene and nonviral reporter gene cassettes were rapidly integrated into the HSV genome in a site-specific and reversible manner by using the P1 phage-based Cre-*lox* recombination system. Shuttle plasmids contained a functional *lox*P recombination site, an expressible form of the bacterial *lacZ* gene, and a copy of the wild-type glycoprotein B (gB) gene or double mutant gB allele containing both a temperature-sensitive (*ts*) mutation and a syncytium (*syn*)-forming mutation. A recipient viral genome, KΔT::*lox*1, was constructed from the HSV type 1 (*syn*) gB-deficient mutant virus, KΔT, by marker transfer of the *lox*P recombination site into the viral thymidine kinase locus. Shuttle plasmids of up to 12.9 kb in length were recombined with high efficiency (11 to 20%) into the KΔT::*lox*1 genome in cell-free, Cre-mediated recombination reactions. Expression of a functional wild-type or double mutant gB polypeptide complemented the nonfunctional polypeptide expressed from the deleted, normal gB locus and allowed production of either wild-type or Syn⁻ plaques on Vero cells. The latter recombinant virus was also *ts* for growth. The ability to express viral genes from plasmids which can be shuttled into and out of the HSV genome in cell-free recombination reactions makes this a powerful method for performing genetic studies of the biologic properties of viral gene products.

Herpes simplex virus type 1 (HSV-1) belongs to a family of animal viruses typified by having large, complex DNA genomes (100 to 250 kb) (for a review, see reference 25). HSV-1 contains approximately 72 genes encoding proteins which may be classified as providing either essential functions for infectious-particle production in cell culture or accessory functions which contribute to viral pathogenesis and extend the virus host cell range during natural infections in vivo. Although the entire HSV genomic sequence is now known (21–23), the biological properties and roles in replication for many of these viral functions have not yet been elucidated.

The production and characterization of HSV mutants have provided the central approach to uncovering the functional roles of individual genes in carrying out different phases of the complex virus life cycle. The principal strategy has been to genetically alter cloned viral DNA segments and then study the affected biologic features of the mutant products either in transient transfection experiments or in infections after marker transfer of the mutant gene into the viral genome by homologous recombination. While transient assays can be useful for the initial characterization of mutant constructs, many experimental approaches require that mutant genes be transferred into the viral genome (3, 7, 9, 11, 12, 29) for analysis of their impact in complex settings such as infection of cell cultures or animals. Genetic studies which rely on marker transfer procedures are usually slowed by the requirement for prior characterization of the mutant construct and by a low yield of recombinants. As a consequence, this approach is rather laborious, particularly for experiments requiring the analysis of large panels of mutant constructs. Moreover, sequences transferred into the viral genome by homologous recombination are not directly recoverable but must be recloned prior to further characterization or manipulation. A more rapid method for introduction of mutant constructs into the HSV genome, which would permit the identification of relevant mutations that result in a particular phenotype, would greatly facilitate the genetic and biochemical characterization of these viral genes and their products.

Work by Sauer et al. (28) demonstrated that plasmid DNA could be efficiently inserted into a herpesvirus genome in an in vitro recombination reaction. By taking advantage of the Cre-*lox* site-specific recombination machinery of bacteriophage P1 (2, 16, 33), a plasmid containing the 34-bp *lox*P recombination site, but completely lacking in homologous herpesvirus sequences, could be specifically inserted at a *lox*P site engineered into the pseudorabies virus genome. The inserted plasmid disrupted the nonessential gIII glycoprotein gene. Recombination was efficient and could be accomplished in a cell-free reaction mixture consisting of only the viral and plasmid DNAs and the Cre recombinase. Transfection of the reaction mixture into suitable eukaryotic cells allowed for the rescue of infectious recombinant virus. Recombination efficiencies of at least 8% were reported, and the inserted plasmids were stably maintained in the viral genome. Furthermore, because the Cre recombination reaction is reversible, the inserted shuttle plasmid could be recovered from the recombinant viral genome by a Cre-

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mediated intramolecular recombination reaction and by propagation after transformation of *Escherichia coli*.

In this report, we describe how the Cre-*lox* shuttle vector strategy was adapted for complementation of an HSV-1 mutant with the essential glycoprotein B (gB) gene deleted. Wild-type and mutant copies of the gB gene were efficiently shuttled into and out of a gB$^-$ *lox*P recipient virus genome. Recombinant viruses were identified on the basis of their blue-plaque phenotype derived from expression of a *lacZ* reporter gene present within the inserted shuttle plasmid. Expression of functional gB polypeptides from the inserted plasmids complemented the gB$^-$ defect of the recipient virus. Insertion of a temperature-sensitive (*ts*) and *syn* double mutant gB allele conferred both mutant phenotypes on the recombinant virus. These studies provided evidence that the Cre-*lox* shuttle system will be useful for the genetic analysis of the roles of both viral genes as well as *cis*-acting genomic elements which function during virus replication.

MATERIALS AND METHODS

Cells and virus strains. Vero cells and the D6 cell line were maintained in Eagle's minimum essential medium (MEM) (GIBCO Laboratories, Gaithersburg, Md.) supplemented with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), the nonessential amino acids, and 5% newborn calf serum (GIBCO Laboratories). D6 cells, which were previously derived by transforming Vero cells with pKBXX (8) and pSV$_2$neo (31), express HSV gB-1 upon infection and complement the growth of gB$^-$ viruses (8). D6 cells were maintained in culture medium supplemented with 200 μg of the antibiotic G418 (GIBCO Laboratories) per ml. For transfection experiments, the cells were maintained in MEM supplemented with 5% fetal calf serum (GIBCO Laboratories) instead of newborn calf serum.

KΔT is a derivative of HSV-1 (strain KOS) carrying a 969-bp *Bst*EII deletion within the essential gB gene and expresses an internally deleted, nonfunctional gB polypeptide which is secreted into the medium (8). Both KΔT and the complementing cell line, D6, were kindly provided by S. Person. KΔT and KΔT::*lox*1 (described below) were propagated, and their titers on D6 cells were determined at 37°C. Cre-mediated recombinants derived from KΔT::*lox*1 were first isolated on D6 cells and then grown, and their titers on Vero cells were determined at 37°C. Recombinants containing the *ts* form of gB were tested for this phenotype by plaque assays on Vero cells at 34 and 39°C. All recombinant virus stocks were derived from plaque-purified isolates.

For plaque assays or individual plaque isolations, serial dilutions of virus stocks were inoculated onto confluent monolayers of the indicated cell line which were subsequently incubated under methylcellulose (0.5% methylcellulose in MEM supplemented with 10 mM HEPES and 2% newborn calf serum) at 37°C until plaques developed. The cultures were then stained either with crystal violet (1% crystal violet in 50% ethanol) or with Bluo-gal (halogenated indolyl-β-D-galactoside) for β-galactosidase (β-gal) activity (see below), and when appropriate, virus from individual blue plaques was isolated for subsequent purification.

Bacterial strains. Plasmids were isolated and propagated in *E. coli* strains DH5α (15) or GM119 (gift from D. Oxender). Plasmid-transformed bacteria were grown in Luria broth (10 g of tryptone per liter, 5 g of yeast extract per liter, 171 mM NaCl) supplemented with 75 μg of ampicillin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml or on L agar plates containing ampicillin.

Construction of plasmids. All cloning steps and plasmid propagations were performed by standard procedures (15, 17, 27). Enzymes were purchased from GIBCO-BRL, Inc., or Boehringer Mannheim Biochemicals and used as specified by the manufacturers. pLink760 (34), pON1 (32), pKBXX (8), and pBS64 and pBS65 (28) have all been described previously. pUCXI (19) contains the HSV-1 *Bam*HI P fragment containing the complete thymidine kinase (*tk*) locus subcloned from pX1 (18) into pUC9. pJG101 was constructed from pUCX1 by inserting a 354-bp *Nae*I-*Sma*I fragment containing a functional *lox*P site from pBS64 into the unique *Sna*BI site within the coding sequence for the *tk* gene. This 354-bp insertion occurs between residues 110 and 111 of the TK protein and was expected to abrogate TK activity. A 270-bp *Bam*HI-*Pvu*II fragment from pBS65, including a functional *lox*P site, was inserted into pBSM13$^-$ (Stratagene, La Jolla, Calif.) between the *Bam*HI and *Sma*I sites, creating pBS*lox*P. pIEP*lacZ* was constructed by subcloning a 763-bp *Bgl*II fragment from pLink760 into the unique *Bgl*II site of pON1, placing a promoterless cassette containing the bacterial *lacZ* gene under the control of the human cytomegalovirus immediate-early promoter (HCM VIEP). The HCMVIEP-*lacZ* gene cassette from pIEP*lacZ* was inserted at the unique *Bam*HI site of pBS*lox*P as a 4.7-kb *Bam*HI fragment, creating pJG103. Finally, to construct pJG108 (see Fig. 3), pJG103 was partially digested with *Bam*HI, the linear unit-length plasmid fragment was gel purified, and a 3.7-kb HSV-1 *Bam*HI-*Bcl*I fragment containing the entire gB-1 (strain KOS) gene from pKBXX was inserted in the orientation shown (see Fig. 3). pJG102 (see Fig. 3) was constructed in a similar fashion from pBS*lox*P by inserting (i) the *ts syn* gB-1 allele from the HSV-1 mutant *ts*B5 as a 4.9-kb *Kpn*I fragment (0.346 to 0.377 map units) at the pBS*lox*P *Kpn*I site and (ii) the 4.7-kb *Bam*HI HCM VIEP-*lacZ* gene cassette from pIEP*lacZ* at the pBS*lox*P *Bam*HI site.

Viral DNA isolation. Viral DNA was isolated by a modification of standard methods for high-molecular-weight eukaryotic DNA isolation (4, 14). Confluent Vero cell monolayers were infected at a multiplicity of infection of 5. At 24 h postinfection, virions free in the medium and released by freeze-thawing cells were combined and pelleted at 75,000 × *g* for 40 min. The virion pellet was resuspended in lysis solution (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.6% sodium dodecyl sulfate, and 0.25 mg of proteinase K per ml) and incubated at 37°C for 8 to 12 h. DNA was multiply extracted first with phenol-chloroform (1:1) and then with chloroform, precipitated with isopropanol, and resuspended in TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) buffer.

Marker transfer of *lox*P into KΔT. The *lox*P site in pJG101 was marker transferred into the *tk* locus of the KΔT genome by homologous recombination after cotransfection of viral and plasmid DNAs into subconfluent D6 cells as described by Graham and van der Eb (13) and modified by Homa et al. (18). Because marker transfer of the *lox*P fragment would interrupt TK expression, transfection lysates were enriched for TK$^-$ viruses (24, 26) by serial propagation at low multiplicity of infection (<0.001) on D6 cells in MEM supplemented with 100 μg of thymine 1-β-D-arabinofuranoside (araT) (Sigma Chemicals, St. Louis, Mo.) per ml. The enriched TK$^-$ stock was used to inoculate D6 cell monolayers, individual plaques were isolated, and small virus stocks were grown on D6 cells in microtiter plates. Infected cell lysates were tested for the *lox*P insert by DNA dot blot assay with the *Nae*I-*Sma*I *lox*P fragment from pBS64 as probe. A recombinant virus, KΔT::*lox*1, containing the *lox*P insert,

was purified by repeated rounds of limiting dilution and dot blot analysis. The *lox*P insertion into the *tk* locus was confirmed by Southern blot analysis (see Fig. 2) (30). Nytran filters containing the electrophoretically separated *Eco*RI or *Bam*HI fragment from the indicated DNAs were first probed with a *tk* probe (2,416-bp *Eco*RI fragment from pUCXI) (see Fig. 3), stripped to remove the *tk* probe, and rehybridized with a *lox*P probe (350-bp *Nae*I-*Sma*I fragment from pBS64) (see Fig. 3). All probes used for dot blot and Southern analyses were labeled with a Random Primed DNA Labeling Kit (Boehringer Mannheim Biochemicals).

In vitro Cre-mediated recombination. *lox*P shuttle plasmids were recombined into the KΔT::*lox*1 genome in cell-free Cre reactions under conditions similar to those described by Abremski and Hoess (1) as modified by Sauer et al. (28). The DNA mixture was incubated at 30°C for 30 to 40 min in the presence of Cre buffer (50 mM Tris-HCl [pH 7.5], 33 mM NaCl, 10 mM $MgCl_2$) and 40 ng of purified Cre protein (NEN Dupont Inc., Wilmington, Del.). For intermolecular recombination, reaction mixtures included KΔT::*lox*1 viral DNA and a 20- to 50-fold excess of shuttle plasmid DNA, and polyvinyl alcohol was added to a final concentration of 1.7% to enhance intermolecular recombination (28, 35). Recombination reactions were terminated by heating samples to 65°C for 10 min, and reaction mixtures were transfected into D6 cells to produce infectious virus. When recovery of the shuttle plasmid from viral DNA was required, purified KΔT::108 viral DNA was incubated with Cre in the absence of polyvinyl alcohol and the recovered plasmid was used to transform DH5α by standard protocols (27).

Purification of Cre-mediated recombinant viruses. Recombinant viruses were identified on the basis of their blue-plaque phenotype and purified by plaque purification and limiting dilution. Transfection lysates were diluted and plated at low multiplicity of infection (<0.01) onto confluent D6 cell monolayers. Plaques were tested for *lacZ* activity with Bluo-gal (GIBCO-BRL), and viruses producing blue plaques were picked as an agarose plug and resuspended in phosphate-buffered saline (PBS). Individual plaque isolates were then cloned by limiting dilution on D6 cells in microtiter plates. Viruses were considered pure when 100% of the plaques stained blue through three successive rounds of limiting dilution.

Assays for *lacZ* activity. Two assays were used to detect *lacZ* activity in developed plaques. For isolation of viable virus, cultures were rinsed with PBS (10 mM sodium phosphate [pH 7.1], 136 mM NaCl), overlaid with 300 mg of Bluo-gal–0.5% low-melting-point agarose (GIBCO-BRL) per ml in MEM supplemented with 10 mM HEPES and 5% fetal calf serum, and incubated at 37°C until color developed. When isolation of viable virus was not required, infected cultures were fixed and stained by a histological method with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Boehringer Mannheim Biochemicals). Infected cultures were rinsed with PBS, fixed for 5 min at room temperature in 2% formaldehyde–0.3% glutaraldehyde in PBS, and incubated in a chromophore solution containing 0.1% X-Gal, 5 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 5 mM $K_3Fe(CN)_6$, and 2 mM $MgCl_2$ in PBS. Incubation continued at 37°C until color developed (usually after 30 to 60 min), and the monolayers were again rinsed with PBS.

RESULTS

Direct isolation of Cre-*lox*-derived recombinant viruses on the basis of β-gal reporter activity. The general strategy for using an HSV-1 shuttle vector system based on the bacteriophage P1 Cre-*lox* site-specific recombination machinery is shown in Fig. 1. The system consists of a recipient virus genome and a shuttle plasmid, each containing a functional *cis*-acting *lox*P recombination site, and the Cre recombinase protein. In a cell-free reaction, Cre-mediated recombination occurs directly between the two *lox*P sites (1) such that the entire plasmid is inserted into the recipient viral genome. Because the Cre reaction is reversible, the shuttle plasmid can also be recovered from the viral genome.

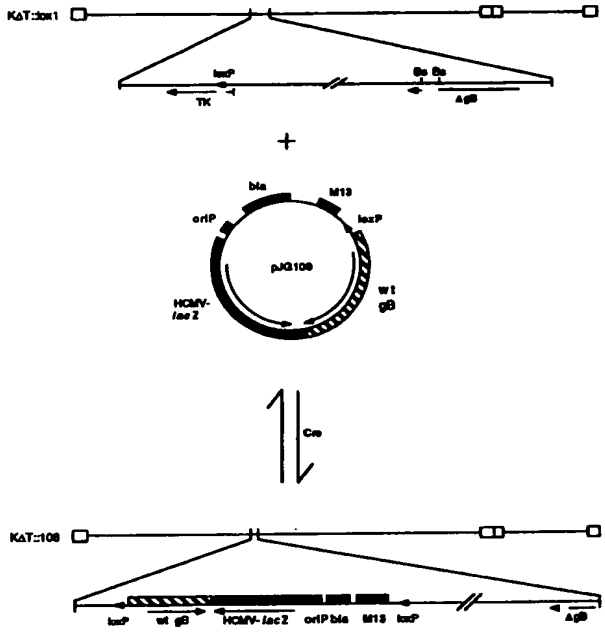

FIG. 1. HSV-based Cre-*lox* site-specific recombination system. The target virus for site-specific recombination, KΔT::*lox*1, was derived from KΔT, and its genome contained (i) a *cis*-acting site-specific recombination site *lox*P from bacteriophage P1 (arrowhead) inserted into the *tk* coding sequence and (ii) a defective gB allele (ΔgB) due to a 969-bp *Bst*EII (Bs) deletion in the gB coding sequence, including the transmembrane domain. In the example shown, the pJG108 shuttle plasmid was recombined into KΔT::*lox*1. pJG108 contained a *lox*P recombination site (arrow), an HCMVIEP-*lacZ* reporter gene cassette for identification of virus recombinants, and the wild-type gB allele from HSV-1 strain KOS. The polarities of both the viral and the plasmid *lox*P sites are shown. pJG108 was inserted into the KΔT::*lox*1 genome at the *tk* *lox*P site in a cell-free Cre-mediated recombination reaction, and infectious virus was produced by transfection of the reaction mixture into D6 cells (8). The recombinant KΔT::108 virus was diploid for the gB gene, containing both the deleted KΔT::*lox*1 allele (ΔgB) and the wild-type KOS allele (wt gB). KΔT::108 also yielded blue plaques in the presence of Bluo-gal or X-Gal because of the HCMVIEP-*lacZ* reporter gene.

A recipient target virus, KΔT::*lox*1, was derived by marker transfer of a functional *lox*P site into the *tk* locus of the gB$^-$ HSV-1 mutant KΔT genome (8). Like KΔT, the KΔT::*lox*1 recipient virus contains a 969-bp *Bst*EII deletion within the essential gB gene and must be propagated on the gB-complementing D6 cell line (8). KΔT::*lox*1 genomic DNA was analyzed by Southern blot (30) to confirm the presence of the *lox*P site within the *tk* coding sequence (Fig. 2).

Shuttle plasmids containing a functional *lox*P site, a re-

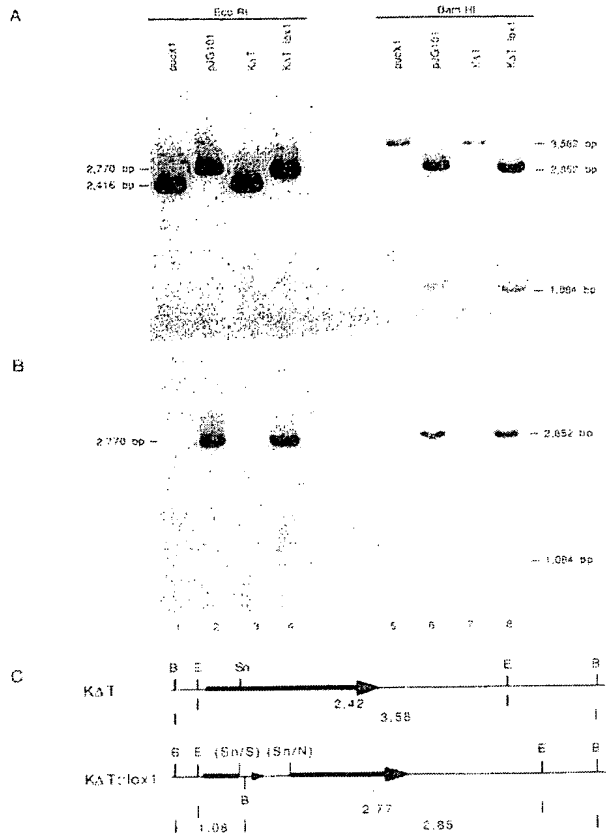

FIG. 2. Southern analysis of KΔT::*lox*1 viral DNA. Plasmid and viral DNAs were digested as indicated, electrophoresed in 1% agarose, and transferred to a Nytran filter (Schleicher & Schuell, Keene, N.H.). The filter was sequentially hybridized with ^{32}P-labeled probes to *tk* (2,416-bp *Eco*RI fragment from pUCX1) (A) and the *lox*P-containing fragment (364-bp *Nae*I-*Sma*I fragment from pBS64) (B). The *tk* probe was quantitatively removed prior to hybridization with the *lox*P probe. (C) Restriction maps of relevant regions. Enzymes used were *Bam*HI (B) and *Eco*RI (E). The positions of the *tk* gene (arrow) and the *tk Sna*BI (Sn) site used to clone the 354-bp *Sma*I (S)-*Nae*I (N) from pBS64 are also shown. Expected restriction fragment sizes in kilobases are also shown.

porter gene consisting of the bacterial *lacZ* gene under the transcriptional control of the strong HCMVIEP-enhancer element, and one of two different alleles of the HSV gB-1 gene were constructed from pBSM13$^-$ (Fig. 3). The reporter gene was included for use as a marker for identifying recombinant viruses containing the inserted shuttle plasmid. pJG108 contained the complete gB gene from the wild-type HSV-1 strain KOS. pJG102 contained the HSV-1 strain *ts*B5 mutant gB gene; the mutant protein product has been shown to be defective at 39°C and to induce polykaryocyte formation, or the syncytial (*syn*) phenotype, in infected cells at 34°C (20).

The pJG102 and pJG108 shuttle plasmids were independently introduced into the KΔT::*lox1* genome in a cell-free Cre reaction. Infectious virus was produced by transfection of the Cre reaction mixtures into the gB-complementing D6 cell line. After 2 days, transfection lysates were harvested

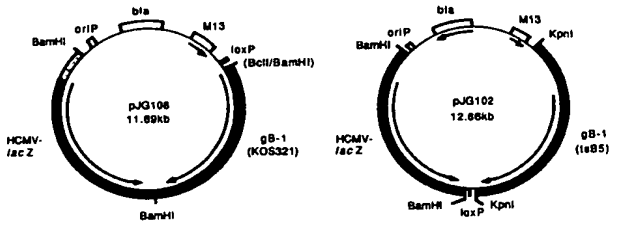

FIG. 3. pJG108 and pJG102 shuttle plasmids. Each plasmid was derived from pBS$^+$ and contains a *lox*P site-specific recombination site, an HCMVIEP-*lacZ* reporter gene, and an HSV-1 gB gene allele as the test gene. The gB genes of pJG108 and pJG102 are derived from HSV-1 strains KOS and *ts*B5, respectively. Restriction sites used to clone the respective fragments are indicated.

and replated on D6 cells and plaques were tested for β-gal activity. Virus from individual blue plaques from each experiment was isolated and subsequently cloned on D6 cells by multiple rounds of limiting dilutions. Viruses were considered pure when 100% of the isolates tested were β-gal$^+$ through three rounds of limiting dilutions. For each virus, insertion of the shuttle plasmid was confirmed by Southern blot analysis (data not shown). Recombinant viruses were named by combining the KΔT:: prefix of the recipient virus with the numeric suffix of the shuttle plasmid to yield KΔT::102 and KΔT::108, respectively.

Cre-mediated recombination is highly efficient. One appealing property of the Cre-*lox* system for introducing DNA into the pseudorabies virus genome was the relatively high recombination efficiency achieved (28). It was therefore of interest to determine the recombination efficiency of the HSV-based system. The presence of the *lacZ* reporter gene on the inserted shuttle plasmid provided a convenient marker for identifying recombinant viruses against a background of nonrecombinant viruses. Since Cre reaction mixtures were transfected into the gB-complementing D6 cells, both the nonrecombinant gB$^-$ KΔT::*lox*1 and the Cre-mediated gB$^+$ recombinants should undergo productive replication with essentially equal efficiencies. Therefore, the percentage of β-gal$^+$ plaques produced on D6 cells from a given transfection lysate provided a reliable estimate of the recombination efficiency of a particular recombination reaction mixture.

DNA from four independent preparations of pJG108 was used in separate Cre reactions with a single preparation of KΔT::*lox*1 viral DNA, and viruses in the resulting transfection lysates were examined for frequencies of recombination. The results are reported as the number of blue plaques per total number of plaques (Table 1). The Cre-mediated

TABLE 1. Cre-mediated recombination efficiency

Plasmid[a]	Dilution[b]	No. of blue plaques	No. of total plaques	% blue plaques[c]
None	−7	0	120	0
pJG108	−5	8	40	20
pJG108	−5	5	36	14
pJG108	−4	26	233	11
pJG108	−5	5	41	12

[a] Cre reactions contained either no shuttle plasmid DNA or DNA from one of four pJG108 plasmid DNA stocks.
[b] Dilution of transfected cell lysate used for blue-plaque assay.
[c] Percent blue-plaque values are (blue plaques/total plaques) × 100.

TABLE 2. Rescue of gB⁻ phenotype of KΔT::*lox*1 by Cre-mediated insertion of pJG108 or pJG102

Virus	Titer (PFU/ml)		D6/Vero efficiency[a]	Plaque morphology	
	D6	Vero		D6	Vero
KΔT::*lox*1	1.8×10^8	0	0	Syn⁺	NA[b]
KΔT::108	8.5×10^8	9.1×10^8	1.07	Syn⁺	Syn⁺
KΔT::102	1.7×10^7	1.8×10^7	1.06	Syn	Syn

[a] For each virus, efficiency is defined as the titer on Vero cells/the titer on D6 cells.
[b] NA, not applicable.

recombination efficiencies ranged from 11 to 20% and averaged about 15%.

Complementation of the gB⁻ phenotype of KΔT::*lox*1. Experiments were carried out to confirm that expression of the polypeptide from the shuttle plasmid would complement the defective gB gene at its native viral locus and that the phenotype of the resulting recombinant virus would reflect the genotype of the plasmid copy of the gene in the *tk* locus. KΔT::108 and KΔT::102 both encode functional gB polypeptides on their respective shuttle plasmids, with KΔT::108 encoding the wild-type gB polypeptide and KΔT::102 encoding the mutant gB polypeptide from HSV-1 strain *ts*B5. The *ts*B5 gB polypeptide includes a *ts* mutation within the external domain (10) and a *syn* mutation (Arg to His) at residue 858 within the cytoplasmic domain (5, 6, 10). Therefore, KΔT::108 and KΔT::102 were used to demonstrate (i) that complementation of the gB⁻ defect of the parental KΔT::*lox*1 virus after insertion of the pJG108 or pJG102 shuttle plasmid occurred and (ii) that the *ts*B5 mutant gB polypeptide encoded by pJG102 conferred both mutant phenotypes (temperature sensitivity and syncytial plaque morphology) on KΔT::102.

In an initial experiment, both the KΔT::108 and the KΔT::102 viruses were shown to replicate normally on Vero cells in plaque assays (data not shown). Therefore, titers of KΔT::108 and KΔT::102 stocks prepared on Vero cells and a KΔT::*lox*1 stock grown on D6 cells were determined on both D6 and Vero cells. As expected, KΔT::*lox*1 developed a titer on the gB-complementing D6 cell line but not on Vero cells (Table 2). In contrast, KΔT::108 and KΔT::102 each grew equally well on either cell line. The plaque morphology of each virus was also examined. KΔT::102 produced the mutant *syn* plaque morphology on both cell lines (Table 2). Therefore, expression of functional gB polypeptides from the inserted shuttle plasmids complemented the deletion within the gB gene at its native viral locus and expression of the allele from pJG102 conferred the *syn* phenotype on KΔT::102. The KΔT::102 recombinant viruses also demonstrated temperature-dependent virus growth. The ratio of KΔT::102 recombinant virus produced at 39°C compared with that produced at 34°C was approximately 5×10^{-4}, an index of temperature sensitivity similar to that of the parent virus *ts*B5. KΔT::108 grew equally well at both 34 and 39°C.

Efficient Cre-mediated recovery of intact shuttle plasmids from KΔT::108 viral DNA. Sauer et al. (28) demonstrated that inserted shuttle plasmid DNA could be excised from the pseudorabies virus genome in a Cre-dependent manner but did not examine the integrity of the recovered shuttle plasmid. If the Cre-*lox* methodology is to be used as a true shuttle vector system, the inserted shuttle plasmids recovered from the viral genome must be intact. The shuttle plasmid was therefore recovered from extracted KΔT::108 viral DNA and compared with the original pJG108 plasmid stock by restriction enzyme analysis. The plasmid was released from purified KΔT::108 DNA in an intramolecular Cre reaction and propagated after transformation of DH5α. Small batches of plasmid DNA (17) were prepared from 10 isolated colonies, and their *Pst*I restriction patterns were compared with that of pJG108. All 10 miniprep DNAs showed restriction fragment patterns that were identical to that of pJG108 (Fig. 4), demonstrating that large shuttle plasmids could be recovered intact from the viral genome by Cre-mediated recombination.

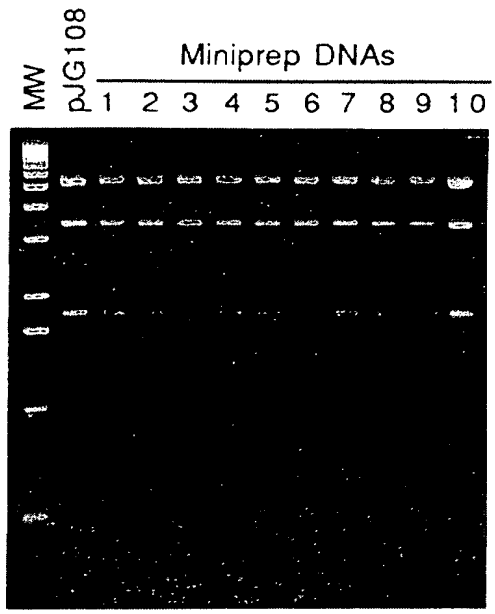

FIG. 4. Cre-mediated rescue of intact shuttle plasmid DNA from KΔT::108 viral DNA. KΔT::108 viral DNA was reacted with Cre recombinase and transformed into DH5α to propagate the rescued shuttle plasmid. Miniprep DNAs were isolated from 10 independent colonies, restricted with *Pst*I, and electrophoresed in 1% agarose. All 10 miniprep DNAs are identical to the pJG108 control.

DISCUSSION

In this report, we describe the adaptation of the Cre-*lox* site-specific recombination system of bacteriophage P1 for use with HSV-1 and investigate the potential usefulness of this approach for the genetic analysis of an essential viral gene. Site-specific recombination mediated by the Cre recombinase protein occurred between two *lox*P sites, one on the shuttle plasmid and the other on the recipient virus genome. A constitutively expressed *lacZ* reporter gene cassette on the shuttle plasmids allowed easy identification of recombinant viruses on the basis of their blue-plaque phenotype. Because Cre-mediated recombination was reversible, the plasmids containing cloned copies of the essential gB gene could be recovered for further analysis by using a second Cre reaction. Recovered shuttle plasmids had a restriction fragment pattern identical to that of the original plasmid. Expression of functional gB polypeptides from the inserted shuttle plasmids at the *tk* locus complemented a

lethal defect in the gB gene. Expression of the *ts*B5 gB allele conferred the *ts* and *syn* phenotypes on the recombinant virus, demonstrating that this approach is useful for testing mutant constructs for altered phenotypes.

The Cre-*lox* shuttle system offers a number of advantages for detailed genetic analyses compared with a strategy based on marker transfer. (i) Since recombination occurs through the 34-bp *cis*-acting *lox*P site, no homologous viral flanking sequences are required to facilitate recombination, minimizing cloning steps that may be needed for marker transfer. Moreover, this site-specific recombinant event avoids potential problems related to rearrangement of the integrated DNA which sometimes occurs in marker transfer experiments. This is particularly important in experiments involving the insertion of large DNA fragments. Although the Cre-*lox* approach does introduce plasmid DNA sequences along with the test gene(s), the function of either the reporter or the gB gene was not affected by the presence of these nonviral sequences. (ii) The recombination frequencies observed for the HSV Cre-*lox* system were even higher than those reported for the pseudorabies virus-based system and are as much as 1 order of magnitude (10 to 20% versus ≤2%) greater than those typically observed in marker transfer experiments. The constructs contained both a complete test gene and a reporter gene such that recombinants can be readily detected and purified on the basis of reporter gene expression. Also, in these studies, the expected complementation of the deleted gB allele by the plasmid-encoded wild-type and *ts*B5 gB proteins could have been used as a means of selecting for recombinants by transfection of the Cre-mediated recombination reaction mixtures directly into noncomplementing Vero cells. (iii) Unlike DNA introduced by marker transfer, genes introduced on shuttle plasmids are directly recoverable from the viral genome in a cell-free Cre reaction without additional cloning steps and are thus readily available for further analysis. This is particularly important for studies requiring the generation and testing of a large panel of mutants. For example, randomly mutagenized genes can be introduced into the viral genome and tested for a particular phenotype such as syncytial plaque morphology, and then only those mutant constructs demonstrating the desired phenotype may be recovered from plaque-purified viral DNA for analysis by DNA sequencing. Thus, the ability to easily recover inserted shuttle plasmids from the viral genome should make a prior detailed analysis of genetic constructs unnecessary. (iv) Finally, the Cre-*lox*-based recombination system should be useful for viral systems for which marker transfer procedures are currently difficult or unavailable. These include the cytomegaloviruses, Epstein-Barr virus, and varicella-zoster virus.

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The Kinetics of Adenovirus Recombination in Homotypic and Heterotypic Genetic Crosses

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The kinetics of recombination between temperature-sensitive mutants has been studied in both homotypic (Ad5 × Ad5) and heterotypic (Ad5 × Ad2⁺ND1) crosses. There is a significant increase in the recombination frequency during the rise period of viral replication in a single-step replication cycle. This observation suggests that adenovirus DNA molecules can undergo progressive rounds of recombination before assembly into virions. To examine this possibility further, advantage was taken of the serotype-specific restriction endonuclease sites and polypeptide sizes of Ad5 and Ad2⁺ND1 to enumerate and to localize the sites of crossing over in ts⁺ recombinants isolated early and late in a heterotypic infection. The late recombinants exhibited, on average, more crossovers per genome than did the early recombinants. This finding is predicted if multiple rounds of recombination take place in some genomes. Using blot hybridization with specific probes, the production of recombinant molecules in the intracellular DNA replicating pool has been followed. Recombinants were found before the rise in infectious virus and increased in frequency relative to the parental molecules throughout the exponential period. These data confirm and extend the genetic observations, which were made on a selected set of infectious virus.

INTRODUCTION

Adenoviruses undergo extensive recombination during the course of a productive mixed infection in cultured animal cells (reviewed by Ginsberg and Young, 1977). Since the adenovirus genome is a single linear DNA duplex, genetic information from molecules of parental genotype must be exchanged by mechanisms as yet undefined, to generate recombinant molecules. We are examining the biological and molecular characteristics of adenovirus recombination to define the functions responsible for such exchange on the assumption that discoveries with the relatively simple and manipulatable viral genome may prove to be important in understanding homologous DNA exchange in eucaryotic cells.

We have chosen to use adenoviruses to examine genetic recombination because they offer a number of distinct technical advantages.

(i) Recombination frequencies (r.f.) between particular genetic markers vary from less than 0.1% to as great as 25%. Thus the effects of a variety of experimental treatments upon r.f. can be evaluated easily. This contrasts with SV-40, where low r.f.s are found (Dubbs *et al.*, 1974).

(ii) The genome is extensively mapped with a wealth of genetic markers and restriction endonuclease sites, and accurate map locations are available for both early and late gene products (Chow *et al.*, 1977; Lewis *et al.*, 1975, 1976, 1977). This allows recombination to be examined over large stretches of the genome, and, in certain cases, fine structure mapping is possible.

(iii) Adenoviruses of closely related serotypes recombine efficiently, but have distinguishable restriction endonuclease sites, thus allowing the DNA structures of recombinants to be examined and the positions of crossovers to be delimited (Grodzicker *et al.*, 1974; Sambrook *et al.*, 1975; Hassell and Weber, 1978). Previously, this

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technique has been used as a mapping tool to locate the physical positions of temperature-sensitive and host range mutations but, as shown in this paper, it has potential for studying the process of recombination itself. In addition, some polypeptides are type specific with respect to antigenicity and mobility on SDS–polyacrylamide gels, allowing the origin of the polypeptide products specified by recombinant genomes to be examined, and the physical locations of the respective genes to be deduced (Mautner *et al.*, 1975; Grodzicker *et al.*, 1977). Conversely, knowing the positions of the genes, we can delimit the positions of crossovers, thus yielding further information on the sites and numbers of exchanges in a particular experiment.

We have taken advantage of the characteristics listed above to study the time course of recombination. It is of considerable importance to know whether or not recombination is a continuous process overlapping in time with the replication of viral DNA and the assembly of new virions. Such information helps to define the kinds of molecules that can interact to produce recombinants. In bacteriophages, contrasting recombination kinetics have been found and have been related to the mode of DNA synthesis. Thus in the T-even series, where recombination and DNA replication are intimately related, there is a steady increase in recombination frequency during the period of active DNA synthesis (Doermann, 1958; Levinthal and Visconti, 1953). This agrees with the population theory of Visconti and Delbrück (1953), in which DNA molecules undergo multiple rounds of mating with a consequent increase in the chance of any two genetic markers recombining. On the other hand, recombination in ϕ X174 is restricted to that period of the DNA replication process when parental replicative forms are present (Doniger *et al.*, 1978; Benbow *et al.*, 1975), and thus r.f. does not increase during the rise period of viral assembly.

In adenoviruses, the frequency of recombination increases throughout the course of virus replication (cited in Williams *et al.*, 1974 and see Fig. 1). To pursue the mechanism of generation of recombinants, we have examined the progeny of heterotypic crosses to determine whether the structure of *ts*$^+$ recombinants, isolated at early and late times postinfection, exhibited different crossover patterns as ascertained by the distribution of type-specific restriction endonuclease sites. Using the Southern blotting technique, with specific probes, we have demonstrated that the events monitored by selecting recombinant virus progeny are mirrored by the changes seen in viral DNA structures present at various times postinfection.

MATERIALS AND METHODS

Viruses. The map positions of the three human type 5 adenovirus temperature-sensitive mutants are as follows.

H5*ts*1 lies between 70 and 73 in the gene specifying the 100K polypeptide (Arrand, 1978; Frost and Williams, 1978; Grodzicker *et al.*, 1977).

H5*ts*2 has been mapped between 50 and 59 (Sambrook *et al.*, 1975; Frost and Williams, 1978) and immunological data strongly suggest that it lies within the hexon gene (Mautner *et al.*, 1975).

H5*ts*142 fails to complement H5*ts*9 (Cheng and Ginsberg, manuscript in preparation) a mutant that almost certainly lies within the fiber gene (Grodzicker *et al.*, 1977; Mautner *et al.*, 1975; Sambrook *et al.*, 1975) and at the nonpermissive temperature, the fiber capsomer does not react with antifiber capsomer antiserum (Cheng and Ginsberg, in preparation). These data, together with the structures of heterotypic recombinants discussed in this paper, are consistent with the suggestion that the mutation lies in the fiber gene.

Ad2$^+$ND1*ts*4 is a mutant of the nondefective Ad2-SV-40 hybrid ND1 (Kelly and Lewis, 1973). Physical mapping data from heterotypic recombination analyses place the *ts* lesion between positions 0.69 and 0.71 on the genome (Grodzicker *et al.*, 1977; Sambrook *et al.*, 1975). This mutant was kindly provided by Dr. Terri Grodzicker, Cold Spring Harbor Laboratories.

All viruses were used as passage three stocks following at least two rounds of plaque purification.

Cells. KB cells in spinner or monolayer culture were used for growing stocks of virus, for the fluorescent focus assay (Lawrence and Ginsberg, 1967), and for most of the recombination experiments. HeLa cells, obtained from Dr. Marshall Horwitz, Albert Einstein College of Medicine, were used for the plaque assay (Williams, 1971). The plaque assay medium was as described (Lawrence and Ginsberg, 1967) but modified by the addition of 0.125 *M* $MgCl_2$. CV1-P cells, obtained from Dr. Ming-Ta Hsu, Rockefeller University, were used in the plaque assay to determine the host range of heterotypic recombinant viruses. $Ad2^+$-ND1 virus produces plaques with equal efficiency on human and monkey cell lines (Lewis *et al.*, 1969) owing to the presence of a helper function specified by the SV-40 inserted sequence. The plaque assay medium for CV1-P cells was as described for HeLa but without the added $MgCl_2$.

Preparation of virus stocks of heterotypic ts^+ recombinants. Well-isolated plaques appearing on CV1-P cells incubated at 39° were picked into 1 ml of infecting fluid (Lawrence and Ginsberg, 1967), frozen and thawed six times to release virus from cells, and 0.2 ml of the resulting samples was used to infect KB cells in 30-mm dishes (Nunc). After incubation at 39° for up to 5 days, cells from each dish were harvested and, following six cycles of freezing and thawing, 0.2 ml inocula were used to infect KB cells in 60-mm dishes (Nunc). After 2 to 3 days incubation at 39°, when total cytopathic effect was observed, the cells were removed from the medium by centrifugation, resuspended in 2 ml of fresh infecting fluid, and frozen and thawed six times to prepare passage 2 stocks.

Preparation of DNA from heterotypic recombinants. Passage 2 stocks were used to infect 2×10^7 KB cells in spinner culture. After 2 days incubation at 37°, the cells were centrifuged and resuspended in 0.01 *M* sodium phosphate buffer pH 7.2. Subsequent extraction of virus followed published procedures (Lonberg-Holm and Philipson, 1969; Pettersson and Sambrook, 1973) except that only one CsCl purification step was employed, namely an overnight centrifugation of the extracted samples in a 1.2–1.4 g/cm^3 density gradient. Isolation of DNA from the virus was as described by Pettersson and Sambrook (1973). After phenol extraction, DNA samples were dialyzed exhaustively against 6 m*M* Tris–HCl buffer pH 7.4, containing 1 m*M* EDTA. The yield of virus DNA from 2×10^7 infected cells was usually between 20 and 50 μg.

Restriction endonuclease analysis of DNA from ts^+ recombinants. *Bam*H-1 (New England Biolabs) digestions were conducted at 37° in a total volume of 50 to 100 μl containing 6 m*M* Tris–HCl pH 7.4, 6 m*M* 2-mercaptoethanol, 6 m*M* $MgCl_2$, 150 m*M* NaCl, between 1 and 2 μg of DNA, and a fivefold excess of enzyme units. After 2 hr digestion, the reaction was stopped by the addition of EDTA to 20 m*M* and then sample buffer was added (0.02% bromophenol blue in gel buffer containing 20% w/v sucrose). DNA was electrophoresed through a 1% agarose gel prepared in 0.09 *M* Tris, 0.09 *M* sodium borate, 0.0025 *M* EDTA titrated to pH 8.0 with acetic acid. After loading 5 to 10 μl of sample (0.1 to 0.2 μg DNA) per well, the DNA was electrophoresed at 20 mA for 2.5 hr. The gels were removed and stained in a 1.0 μg/ml solution of ethidium bromide and the DNA fragment bands were photographed after exposure to irradiation with short-wavelength ultraviolet light (Sharp *et al.*, 1973).

Analysis of polypeptides from recombinant-infected cells. KB cells in 30-mm dishes were infected with fivefold dilutions of passage 2 virus stocks and incubated at 39° for 2 days. The cells were washed twice with phosphate-buffered saline and then resuspended in 3 ml of 0.01 *M* sodium phosphate buffer pH 7.2. Samples, 50 μl, containing approximately 5×10^4 cells, were boiled with 25 μl of 3× concentrated sample buffer (Kauffman and Ginsberg, 1975) for 1 min and then 20-μl samples of dissociated cells were layered onto 7.5% polyacrylamide gels containing sodium dodecyl sulfate. The gel system has been described previously (Kauffman and Ginsberg, 1975). After 3 hr electrophoresis at 100 V, the gel was stained in Coomassie brilliant blue (0.2% Coomassie brilliant blue,

50% methanol, 7% acetic acid) for 1 hr and then destained extensively in 50% methanol, 7% acetic acid. Following destaining, the gel was rehydrated and then vacuum dried on a Hoefer Scientific gel dryer.

Isolation and analysis of DNA from infected cells. KB cells at a concentration of 2×10^6 cells/ml were inoculated with 3.3 FFU of Ad2$^+$ND1*ts*4 and 6.6 FFU of H5*ts*142. After 2 hr adsorption, when ~60% of the virus had adsorbed, the cells were centrifuged, washed with phosphate-buffered saline, resuspended to a final concentration of 2×10^5 cells/ml, and incubated at 32° for 72 hr to measure viral yield by fluorescent focus assay. In addition, samples containing 2×10^7 cells were withdrawn and the cells were centrifuged, washed with phosphate-buffered saline, and stored as frozen pellets. The pellets were resuspended in 40 vol of TNE [10 m*M* Tris–HCl (pH 8.0), 150 m*M* NaCl, 10 m*M* EDTA] and SDS and proteinase K were added to 0.2% and 100 μg/ml, respectively. The lysate was incubated at 37° for 6 hr and then extracted sequentially with buffer-saturated phenol and $CHCl_3$:isoamyl alcohol (24:1). High molecular weight DNA was isolated by mixing the aqueous phase with 2 vol of ice-cold ethanol and immediately spooling out the precipitate that formed. The DNA was washed with 70% ethanol and dissolved in H_2O. The DNA solution was brought to 10 m*M* Tris, 2 m*M* EDTA (pH 7.5), and digested with 40 μg/ml of heat-treated pancreatic RNase for 2 hr at 37°. SDS and proteinase K were added to the solution as above and incubation continued for 2 hr at which time NaCl was added to 200 m*M* and the samples were sequentially extracted with phenol and chloroform:isoamyl alcohol. The purified DNA was resuspended in sterile H_2O and stored at 4°.

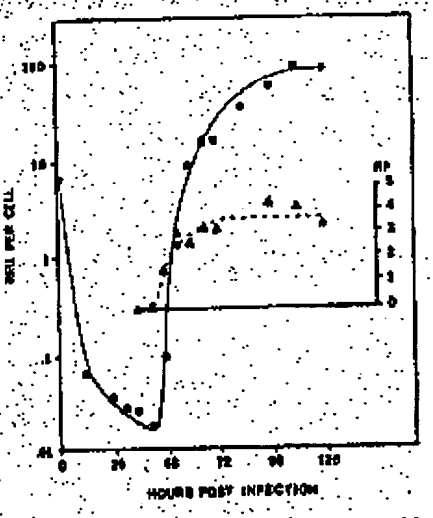

FIG. 1. The kinetics of homotypic recombination. Replica monolayers of HeLa cells containing 3.2×10^6 cells per 60-mm dish were infected with 2.2 plaque-forming units (PFU) of H5*ts*1 and 4.8 PFU of H5*ts*2. After adsorption, the plates were rinsed, treated with antiserum to Ad5, rinsed again, and overlaid with Eagle's medium containing 2% calf serum. The dishes were incubated at 32° and at intervals, individual cultures were harvested, and the cell-associated virus was released and titrated at 39 and 32° on HeLa cell monolayers. Recombination frequency = titer at 39°/titer at 32°C × 2 × 100.

Filter hybridization. DNA from infected cell cultures was digested with *Bam*H-1 as described above and electrophoresed at 65 V on horizontal agarose slab gels (0.8%) in 36 m*M* Tris, 30 m*M* NaH_2PO_4, 1 m*M* EDTA (pH 7.9). The gels were stained with EtBr (1 μg/ml), photographed, and DNA fragments were transferred to nitrocellulose sheets, hybridized, and washed as previously described (Wigler *et al.*, 1979).

RESULTS

The Kinetics of Homotypic Recombination

The recombination frequency (r.f.) between two temperature-sensitive mutants of adenovirus increases during the exponential phase of a one-step replication cycle in HeLa cells (cited by Williams *et al.*, 1974). An example of this phenomenon is shown in Fig. 1. Replicate cultures of HeLa cells in 60-mm plastic dishes were coinfected with H5*ts*1 and H5*ts*2, mutants previously mapped by marker rescue in the 100K and hexon genes, respectively (Frost and Williams, 1978; Arrand, 1978), and analyzed for the presence of recombinants at intervals postinfection. The most pronounced increase in r.f. (from 0.05% at 36 hr to 3.1% at 54 hr) occurred during the first half of the exponential rise in in-

fectious virus. At this time viral-specific DNA synthesis is progressing at a maximal rate (Young, unpublished). Some very small plaques were observed in the 39° assay at times earlier than 36 hr postinfection, but isolates from 12 individual plaques were found to contain virus that was genotypically temperature sensitive, suggesting that the plaques had arisen by complementation on the assay plate, a phenomenon first demonstrated by Ensinger and Ginsberg (1972). Calculation of the number of cells on the assay plate that would be biparentally infected, at the dilutions used, is consistent with this hypothesis. In reconstruction experiments, where *ts*1 and *ts*2 were mixed *in vitro* and assayed at similar dilutions, plaques were seen at 39°. In contrast, 13 of 16 plaques isolated from samples at 54 and 96 hr postinfection were genotypically *ts*$^+$. The growth curves for the two single infections (not shown) paralleled that of the cross, but the frequencies of plaquee at 39° were at all times less than 0.01%.

These data, and essentially similar ones obtained in KB cells with a different pair of Ad5 alleles (Young, unpublished), strongly suggest that recombination is a dynamic process occurring continuously throughout the first half of the exponential phase of virus replication, and is not confined to an initial step in DNA synthesis that would occur during the eclipse phase.

A Temporal Analysis of Heterotypic Recombination

An increase in recombination frequency during the exponential rise is consistent with the postulate that adenovirus DNA molecules undergo multiple rounds of mating during the period of DNA synthesis, but several other possibilities exist. For example, DNA synthesis factories at first are separate, but fuse with time (Boyer *et al.*, 1967; Yamaguchi *et al.*, 1977) and genetic recombination would have to await such fusion if each original factory is established by one input DNA molecule. Alternatively, the enzymes for recombination may be induced asynchronously in different cells so that DNA made and packaged early in some cells will not have had an opportunity to recombine. There are many other possibilities involving asynchronous cellular or viral functions. To explore some of these possibilities, we have examined the production and structure of heterotypic recombinants formed between H5*ts*142, a mutant defective in the fiber polypeptide (Cheng and Ginsberg, in preparation) and Ad2$^+$ND1*ts*4, a mutant defective in the 100K polypeptide (Grodzicker *et al.*, 1977).

The design of the heterotypic cross. The aims of the heterotypic cross were to determine if a temporal increase in recombination frequency occurs, and more particularly, to see if the DNA structures of *ts*$^+$ heterotypic recombinants isolated at early and late times in infection differed. The design of the experiment was chosen with the following criteria in mind. (i) Each *ts*$^+$ recombinant must be clonally unrelated. (ii) The choice of mutants, and of the restriction enzymes involved in analyzing DNA structure, should allow recombination to be observed over wide stretches of the genome. (iii) The isolation of recombinants, especially at early times when very few are present, must be reliable with as little confusing contribution from *ts*$^+$ revertants as possible. The first criterion was achieved by isolating a set of single *ts*$^+$ recombinants from a series of parallel samples withdrawn from an infected spinner culture. The second criterion was limited by the paucity of *ts* markers whose physical locations had been determined accurately and by the lack of different restriction endonuclease sites for more than a few enzymes in any pair of serotypes that can undergo recombination. The choice of H5*ts*142 and Ad2$^+$ND1*ts*4 was made because the map position of the latter was known to lie between 69 and 71 (Grodzicker *et al.*, 1977) while the former has a fiber-defective phenotype and fails to complement *ts*9 (Cheng and Ginsberg, manuscript in preparation) which is known to lie to the right of 86 (Sambrook *et al.*, 1975). These map coordinates in theory allow supernumerary crossovers (those crossovers *other* than the one between *ts*4 and *ts*142) to be distinguished

from position 69 leftward to 0. In practice the leftmost coordinate was at position 29, a *Bam*H-1 restriction site at which Ad5 and ND1 differ. The enzyme of choice for these analyses was *Bam*H-1. The enzyme cleaves ND1 into five distinguishable fragments ranging in percentage molecular weight from 29 to 13% while Ad5 is cleaved into two fragments of 59.5 and 40.5%. (Sambrook *et al.*, 1975). Figure 2 shows the salient map positions of the enzyme cleavage sites and the *ts* markers and the regions in which supernumerary crossovers can occur. Figure 4 reveals that supernumerary crossovers in regions II and III produce DNA fragment patterns distinguishable from that produced by a single crossover between the *ts* markers and from those produced by one or both parents. Supernumerary crossovers in region I are not detectable. The third criterion mentioned above, namely the reliability of the ts^+ selection technique, was met by selecting recombinants on monkey CV1-P cells. ND1*ts*4 is not leaky nor does it revert frequently while H5*ts*142 has an appreciable reversion frequency on HeLa cells [as high as 1×10^{-4} (Young, unpublished)]. Thus, any plaques appearing at 39° on CV1-P will almost certainly arise from infection with a recombinant containing the SV-40 sequences necessary for growth on monkey cells, the *Bam*H-1 site at 85 percent in these sequences (Sambrook *et al.*, 1975), and lacking both *ts* sites. Revertants of H5*ts*142 on the other hand will not replicate efficiently on CV1-P cells.

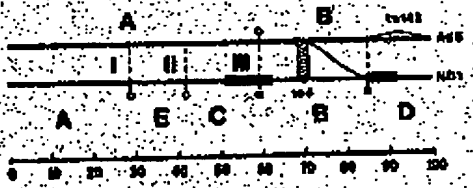

FIG. 2. Physical maps of the genomes of H5*ts*142 and Ad2⁺ND1*ts*4. *Bam*H-1 sites (▽) define five digestion products (A–E) for ND1 and two products (A', B') for Ad5. The genes for hexon (51.9 to 68.2) and fiber (86.2 to 91.5) are shown (▨) (Chow *et al.*, 1977). The approximate locations of *ts*4 and *ts*142 are indicated (see Materials and Methods). The SV-40 sequence present in ND1 is close to the left of the fiber gene. A crossover (╲) is depicted that will generate a ts^+ recombinant that can replicate on CV1-P cells at 39°.

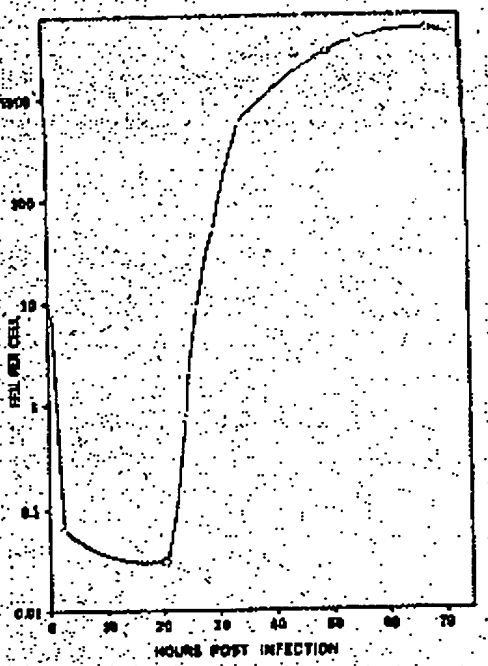

FIG. 3. The growth curve for a heterotypic mixed infection. KB cells, at 1.0×10^6 cells/ml, were inoculated with 8 FFU of H5*ts*142 and 9 FFU of AD2⁺ND1*ts*4. After 2 hr adsorption, the cells were centrifuged, a sample of the supernatant was taken to measure unabsorbed virus (~50% in this experiment), and the cells were resuspended in fresh spinner medium at a concentration of 2×10^5 cells/ml. The culture was maintained at 32° and at intervals, samples were withdrawn and virus was assayed by fluorescent focus assay on KB cells at 32°. At approximately 22, 24, 26, 72 hr postinfection, 40 replicate samples were withdrawn and, after the growth curve had been determined, the 24-and 72-hr samples were plated on CV1-P cells and incubated at 39° for the isolation of putative ts^+ recombinants.

The DNA structures of heterotypic ts^+ recombinants. Spinner KB cells were infected with H5*ts*142 and Ad2ND1*ts*4. Samples were removed at intervals and total infectious virus was measured at 32° by fluorescent focus assay. The growth curve is illustrated in Fig. 3. At 24 and 72 hr postinfection, 40 individual samples were removed, and appropriate dilutions were plated on CV1-P cells at 39° to select for ts^+ recombinants. Plates that contained very few plaques were used for the isolation

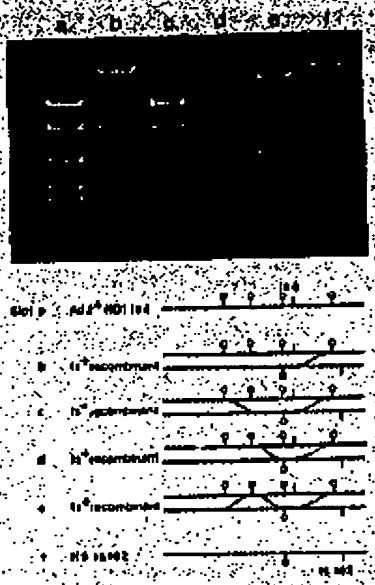

FIG. 4. Electrophoresis of *Bam*H-1 digestion products of DNA from the parents and representative *ts*$^+$ recombinants, obtained from the heterotypic cross H5*ts*142 and Ad2$^+$ND1*ts*4. The diagrams below the gel are interpretations of the approximate locations and orientations of the crossovers present in each *ts*$^+$ recombinant, deduced from the corresponding fragment pattern. (↑) *Bam*H-1 site; (|) approximate *ts* site.

of putative *ts*$^+$ recombinant virus clones. Great care was taken to sample plaques of all sizes and morphologies as a priori there is no way of knowing whether or not certain recombinant classes give rise to distinctive plaque types. A representative set from the 24-hr samples was tested for plaque-forming ability at 39 and 32° on HeLa cells. Only 12 of 40 individual plaque isolates tested plaqued efficiently at 39°. The remainder may have arisen by complementation on the assay plate, just as in the early samples from the homotypic cross. The early time samples were replated and a total of 28 *ts*$^+$ isolates, each from a separate sample, was obtained. Among the 72-hr samples, 31 of the 40 gave genotypically *ts*$^+$ isolates after the first selection.

Small stocks of purified virus were prepared from each *ts*$^+$ isolate and viral DNA was extracted, cleaved with *Bam*H-1, and the fragments were displayed on an agarose gel. Table 1 gives the cumulative data for all the *ts*$^+$ isolates examined and Fig. 4 (reproduced from Young and Fisher, 1980) shows the various classes of fragment pattern observed in this experiment with interpretations of the disposition of crossover points. It is pertinent to note that the r.f. was 10-fold higher in the sample isolated at 72 hr when compared with that isolated at 24 hr.

Comparing recombinants isolated from 24 hr and 72-hr samples, several conclusions may be drawn. (i) The later set contains representatives of four classes of recombinant while the earlier set has only two. If the numbers of representatives of each class are compared in a 2×4 contingency table, the increase from early to late times in the occurrence of supernumerary crossovers is seen to be highly significant ($P = 0.004$). (ii) At late times the majority of recombinants are formed by single crossovers but there is a greater than expected frequency of multiple crossovers, if recombination frequency is based simply on physical distance between the genetic markers. The recombination frequency between *ts*4 and *ts*142 is only 0.34% yet approximately 38% of the recombinants contain supernumerary crossovers. (iii) As expected, all recombinants

TABLE 1

SUMMARY OF *Bam*H-1 RESTRICTION ENDONUCLEASE ANALYSIS OF DNA FROM *ts*$^+$ ISOLATES FROM THE CROSS H5*ts*142 × Ad2$^+$ND1*ts*4[a]

Time of sample (hr)	R.F. (%)	No. of isolates with No. of crossovers			
		1	2a	2b	3
24	0.034	26	2	0	0
72	0.34	19	7	4	1

[a] The fragment patterns produced by the various types of crossover are diagrammed in Fig. 4. Class 2a gives A, E + C, B, D and 2b gives A, B, C, D, E. Comparing the frequencies of the various crossover classes at early and late times, using Fisher's exact test for small numbers, the probability that the samples came from the same statistical population is 0.004. That is, the frequency distribution at 72 hr is significantly different from that at 24 hr.

contained the endonuclease site at 85 percent in the SV-40 sequence inserted in ND1.

The polypeptides specified by heterotypic ts⁺ recombinants. The hexon and fiber polypeptides specified by Ad5 and Ad2⁺ND1 differ in mobility on SDS–polyacrylamide gels. We have taken advantage of this observation to investigate whether or not the heterotypic recombinants analyzed by *Bam*H-1 restriction can be distinguished further by the hexon and fiber polypeptides they specify. In addition, this analysis can determine whether the few putative recombinants that have an ND1 fragment pattern (Table 1, class 2b) are indeed recombinants and not revertants of *ts*4.

Passage 2 stocks of individual *ts*⁺ isolates were used to infect KB cells at 39° and after 2 days the cells were harvested, washed in phosphate-buffered saline, and extracts were electrophoresed in 7.5% SDS–polyacrylamide gels. Figure 5 shows such a gel after staining the polypeptides with Coomassie brilliant blue. All of the *ts*⁺ recombinants had hexon polypeptides with a mobility characteristic of the Ad5 parent and fibers characteristic of the ND1 parent. The *ts*⁺ isolates with an ND1 DNA fragment pattern were clearly recombinant, as they specified Ad5 hexon. These results are consistent with the crossover interpretations presented in Fig. 4 and help to localize the positions of some of the supernumerary crossovers. Thus an extra crossover in region III (Fig. 2), as defined by the *Bam*H-1 site at 42 and the site of *ts*4, must lie to the left of position 62.2, the rightward boundary of the Ad2 hexon (Chow *et al.*, 1977). If the cross-over occurred to the right of this site, then the recombinant would specify an Ad2 hexon. The data also strongly support the notion that the lesion in H5*ts*142 lies in the fiber gene since all the recombinants specify ND1 fiber.

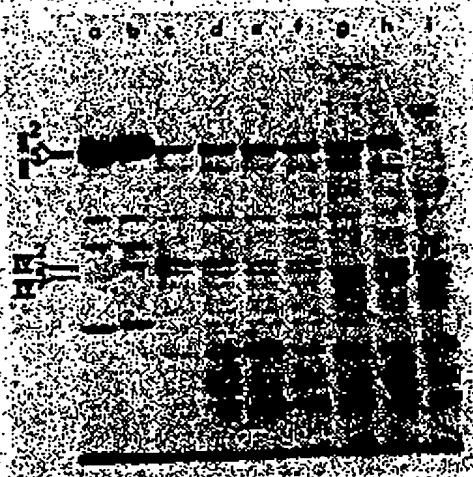

FIG. 5. SDS–polyacrylamide gel electrophoresis of infected cell extracts. KB cells were infected with representatives of each *ts*⁺ recombinant class described in Fig. 4 and Table 1 and with the parents. Samples were prepared as described under Materials and Methods. The lanes contain the following: c to f, infected-cell extracts from *ts*⁺ recombinants; a and b, purified virion markers from H5*ts*142 and Ad2⁺ND1*ts*4, respectively; g and h, extracts from *ts*142 and *ts*4, respectively; lane i, uninfected cell extract. II^1, II^2 refer to the hexon polypeptide specified by Ad2⁺ND1*ts*4 and H5*ts*142, respectively. IV^2, IV^1 refer to the respective fiber polypeptides.

Mixed plaques. DNAs from 59 *ts*⁺ isolates were examined by *Bam*H-1 digestion. In 6 cases, (2 from the 24-hr samples and 4 from the 72-hr samples), the ethidium bromide staining pattern of *Bam*H-1-digested DNA revealed fragments characteristic of both patients, even after exhaustive digestion. This suggests that the plaques arose by complementation on the assay plate and that this mixed population had persisted even through the first passage of growth at 39°, when the m.o.i. was presumably much less than 1. One late sample had a fragment pattern characteristic of ND1 but with fragments C and E underrepresented. The origin of this pattern was not immediately obvious. The virus in this plaque isolate and in one of the putative complementing plaques were examined in detail by replating the plaque sample on HeLa cells at 32° and isolating individual subclones. Under these conditions, all classes of parental and recombinant virus will replicate. Each subclone was tested for the ability to replicate on HeLa cells at 39 and 32° and on CV1-P cells at 32°. The plaque that was suspected of containing both parental viruses contained virus of the appropriate phenotypes and also some recombinants that

grew at 39° on HeLa cells and 32° on CV1-P cells. These *ts*$^+$ recombinants presumably had a strong selective advantage at 39° during the growth of the stocks from the original plaque isolate. The other plaque contained virus all of which grew at 39° on HeLa cells and on CV1-P cells at 32°, strongly suggesting that the plaque contained *ts*$^+$ recombinants only. Fifteen subclones were grown up and their DNAs examined by cleavage with *Bam*H-1. Eleven of them contained DNA structures, A, E + C, B, D, while four had structures A, B, C, D, E. This admixture explains the deviation from equimolarity observed in the original DNA digestion. The presence of these two genotypes within a single plaque could arise from a clump of two different rare *ts*$^+$ viruses or from the segregation of a region of heteroduplex DNA, heterozygous for the E·C *Bam*H-1 cleavage site.

The Kinetics of Adenovirus DNA Recombination

The data in the preceding sections have demonstrated that the frequency of *ts*$^+$ recombinant virus increases during the exponential rise period and that the numbers of supernumerary cross-overs in the DNA of these viruses are significantly higher at late times. Genetic analyses require that, following recombination, the DNA be assembled and fully infectious virus be formed. Thus, this analysis may be biased by the failure to detect events that lead to recombinant DNA molecules incapable of being assembled or of replicating in the absence of helper. To circumvent this, we examined the time course of production of recombinant DNA molecules in infected cells by using molecular hybridization. The experimental design of these analyses was to employ specific fragments of Ad2 DNA to probe the structure of the total intracellular DNA pool and to examine the generation and replication of recombinant DNA molecules throughout the course of mixed infection. In this experiment, fragment E was used to probe DNA fragments obtained following *Bam*H-1 cleavage of total infected-cell DNA. This probe will anneal to the E fragment of ND1, the A' of Ad5, and two recombinant fragments that arise following reciprocal crossovers in region II (Fig. 2). The putative recombinant fragments A + E and E + C represent 42 and 30.5% of the genome, respectively. The 42% fragment is unique while the 30.5% fragment comigrates with the parental A band and therefore can only be identified in the pool of replicating molecules by hybridization to the E probe.

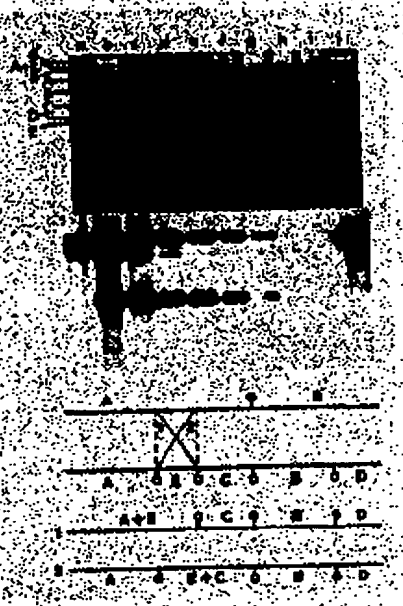

FIG. 6. Analysis of intracellular DNA from the heterotypic cross H5*ts*142 × Ad2$^+$ND1*ts*4. The preparation of the DNA samples is described under Materials and Methods. *Bam*H-1-cleaved DNA, 7.5 μg, was loaded in each lane (b–i). The top panel shows the agarose gel stained with ethidium bromide and photographed under short-wave ultraviolet light. The middle panel is the corresponding autoradiogram, following blot hybridization with a ^{32}P-labeled probe corresponding to fragment E of ND1. The schematic gives the orientations of the crossovers in the E region with the production of specific recombinant fragments A + E and E + C (the right-hand portion of the molecule is shown as B, D regardless of orientation). The digests in the various lanes were from samples isolated at the following times postinfection: (b) 50 hr 45 min; (c) 40 hr 30 min; (d) 38 hr 30 min; (e) 25 hr 30 min; (f) 22 hr 35 min; (g) 19 hr 30 min; (h) 16 hr 40 min; (i) 3 hr 0 min. Lanes a and j: a DNA digest from a *ts*$^+$ recombinant containing a known A + E recombinant fragment.

The orientation of crossovers is depicted in Fig. 6.

KB cells in spinner culture were infected with *ts*4 and *ts*142 under conditions similar to those used for the genetic analysis and, at intervals, samples were withdrawn to measure the total viral yield and to analyze the pool of DNA molecules. The growth curve was comparable to that shown in Fig. 3 with a rise period beginning at approximately 19 hr postinfection. The DNA was extracted, digested with *Bam*H-1, and equal amounts of the digestion products were electrophoresed on a horizontal agarose gel. The DNA was denatured *in situ*, transferred to nitrocellulose paper, and hybridized to ^{32}P nick-translated fragment E probe. Figure 6 shows an autoradiograph depicting the time course of production of fragments that hybridize to E. Three points stand out clearly. The total amount of adenovirus DNA increases very significantly throughout the rise period (19.5–38.5 hr postinfection); the two recombinant bands are detected as early as 16⅔ hr postinfection (this is detectable on the original autoradiograph) and, qualitatively, appear to increase relative to the other bands; and there is no evidence of bands, which hybridize to E, but migrate with a mobility other than those expected (A′, A + E, E + C, and E).

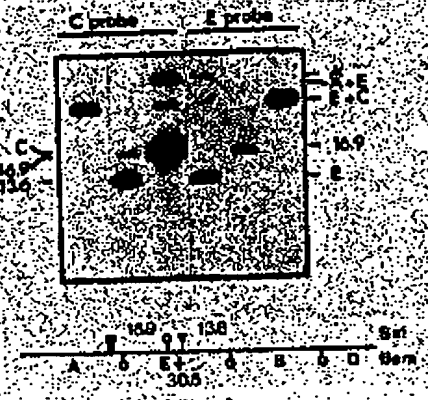

FIG. 7. Analysis of the putative E + C recombinant fragment. DNA from a sample withdrawn at 50 hr 45 min post-infection was cleaved with *Bam*H-1, electrophoresed, and the band with a mobility equivalent to ND1 E + C was isolated and digested with *Sal*-1. The digestion products were electrophoresed, transferred to nitrocellulose, and hybridized with ^{32}P-labeled fragments corresponding to *Bam*H-1 ND1 C or E. The autoradiogram lanes contain the following (from left to right): known *Bam*H-1 E + C fragment; putative *Bam*H-1 E + C fragment digested with *Sal*-1; *Bam*H-1 digestion products from the 50 hr 45 min sample; *Bam*H-1 digestion products from the 50 hr 45 min sample; putative *Bam*H-1 E + C fragment digested with *Sal*-1; known *Bam*H-1 E + C fragment. Below is a schematic with the relevant *Bam*H-1 sites (▽) and *Sal*-1 sites (▽). The *Bam*H-1 site at the E + C junction, deleted in the E + C recombinant fragment, is shown above the line. The approximate fragment sizes of E + C and of the *Sal*-1-digested E + C are also given.

The A + E recombinant band migrates noticeably more slowly than the Ad5 B′ band and can be detected both by hybridization and, as early as 22⅔ hr postinfection, by ethidium bromide staining (Fig. 6). As pointed out above, the mobility of the E + C band is indistinguishable from the A band and although the fragment E probe should not cross-hybridize with A, we wished to confirm that it was detecting a genuine E + C recombinant species. The diagram in Fig. 7 indicates that the restriction endonuclease *Sal*-1 will cleave an E + C recombinant fragment once, yielding two pieces representing 16.9 and 13.6% of the genome. A probe prepared from fragment E will detect only the 16.9% *Bam*/*Sal* fragment, while a probe prepared from fragment C will detect both *Bam*/*Sal* fragments.

To determine if the 30.5% fragment detected by hybridization with the E fragment probe was a genuine recombinant, DNA from a late time sample was digested with *Bam*H-1, the fragments were separated by electrophoresis, and the band corresponding to E + C (and ND1-A) was isolated and the DNA redigested with *Sal*-1. DNAs from a recombinant known to contain the E + C fragment and from a late time sample were cleaved with *Bam*H-1 alone. Samples from the three digestion mixtures were electrophoresed in parallel in two separate sets. The gel was blotted onto nitrocellulose and the filter was cut to separate the two sets. One set was probed with ^{32}P nick-translated E fragment, while the other was probed with C fragment.

The autoradiogram is shown in Fig. 7. As expected, the E probe detected only a 16.9% *Bam*/*Sal* fragment while the C probe detected both the 16.9 and the 13.6% fragments. Both probes detected A′, E + C, and either E or C as expected and only the E probe detected A + E. These results clearly demonstrate that the band that migrates with the mobility of ND1-A and is detected by the E probe is indeed recombinant in region II and lacks the *Bam*H-1 site at the E·C junction.

The data from the blotting experiments fully substantiate the conclusions drawn from the genetic experiments. Furthermore, the technique allows recombinational events to be monitored in the absence of viral assembly and subsequent replication, and will prove to be of great value in situations where such assembly does not happen or has been restricted experimentally.

DISCUSSION

We have employed genetic and molecular techniques to analyze the frequency of recombination and the DNA structure of individual ts^+ recombinants that arise during the course of a mixed infection of animal cells with adenoviruses. Our data demonstrate a temporal change in the recombination frequency (r.f.) during the course of infection, with at least a 10-fold increase in r.f. occurring during the exponential rise period (Fig. 1). In addition, the observed frequency of supernumerary crossovers increases significantly when recombinants isolated at late times are compared with early isolates (Table 1). Examination of temporal changes in the intracellular pool of replicating DNA by blot hybridization with specific probes allowed us to follow the production and replication of recombinant genomes. These analyses have revealed the appearance of recombinant molecules prior to the time at which they could be detected using conventional genetic analyses. Furthermore, the frequency of such molecules compared to the parental forms increases with time. These data, derived from analyses of molecular hybridization experiments, support and extend the genetic data.

The temporal increase in recombination frequency in both homotypic and heterotypic crosses and the increase in recombinant molecules in the intracellular DNA pool are phenomena that have been observed in T-even bacteriophages both by genetic (Doermann, 1953; Levinthal and Visconti, 1953) and physical methods (Kozinski, 1961). In these phages there is temporal overlap in DNA synthesis and virion assembly so that there is a random withdrawal of DNA molecules from a replicating pool. If recombination occurs throughout the period of DNA replication, it follows that DNA molecules withdrawn late from the replicating pool will have experienced, on average, more exchange events than those withdrawn early. This will be revealed by a progressive increase in recombination frequency between any two markers, a so-called "drift to genetic equilibrium" (Doermann, 1953; Levinthal and Visconti, 1953). A similar phenomenon has been observed with herpes simplex virus and may well arise from the same causes (Ritchie *et al.*, 1977). In contrast, the major recombinational events in ϕX174 occur only between parental replicative forms at the beginning of DNA synthesis and before viral assembly begins (Doniger *et al.*, 1973; Benbow et al., 1976). Thus, r.f. does not increase during the exponential rise period. In adenoviruses, as with T-even phages, DNA synthesis and virion formation overlap, since during the exponential rise period, adenovirus-specific DNA accumulates (Fig. 6). Therefore the increase in r.f. and in the abundance of recombinant molecules observed fits the T-phage model discussed here and suggests that adenovirus DNA molecules undergo an increasing number of exchange events as the infection proceeds. In this model, there should be a progressive rearrangement of nonselected genetic markers within a selected set of recombinants. The analysis of supernumerary crossovers at early and late times in infection (Table 1) demonstrates that nonselected restriction endonuclease site markers have been significantly rearranged at late times. Unless the molecular events underlying the production of recombinants change qualitatively with time, the ob-

servation of an increase in supernumerary crossovers at late times strongly suggests that some molecular lineages have experienced multiple recombinational events.

The frequency of multiple crossovers in ts^+ recombinants is greater than would be expected on a random basis. At 72 hr, there are 11 recombinants with double crossovers and one with a triple among the 31 recombinants examined (Table 1). The recombination frequency for the *ts*4 × *ts*142 cross is 0.84% (Table 1) for a molecular distance of some 20% of the genome length. Additional crossovers in the region of the genome from *ts*4 to the *Bam*H-1 site at position 29 should occur at a frequency of 0.68% (0.84 × 40/20) if such crossovers are independent events. Instead, they occur at a frequency of some 42% (13/31 × 100). The excess of supernumerary crossovers can also be seen at 24 hr postinfection, where 7% (2/28) extra crossovers are observed compared with an expected 0.068%. The high frequency of multiple crossovers in a set of ts^+ recombinants has been observed and discussed before in analyses of heterotypic crosses of both adenoviruses (Sambrook *et al.*, 1975) and herpes simplex viruses (Morse *et al.*, 1977). These observations imply that crossover events are *not* random with respect to one another when a selected set of ts^+ recombinants are examined. Thus, it is important to consider whether or not the temporal analysis of supernumerary crossovers is affected by this nonrandomness. The excess of crossovers, over those predicted, is observed at both early and late times but the absolute frequency increases significantly. Thus we conclude that, despite the nonrandomness of the events we are observing, some molecules isolated at late times have had a different recombinational "history" to those isolated earlier. The simplest interpretation of this observation is that some molecules undergo multiple rounds of recombination.

Blot hybridization analysis of the production of recombinant molecules has proved to be a valuable tool. It has provided molecular evidence for the temporal increase in specific recombinant molecules and has detected such molecules at a point during the replication cycle at which genetic recombinants cannot be observed, namely in eclipse (Fig. 6, 16½ hr postinfection). This indicates that the technique will be useful to detect recombinant molecules which arise under naturally occurring or experimental conditions in which DNA has entered the cell but in which infectious virus is not produced.

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CHAPTER 5

Multiplication of Viruses

An Overview

Bernard Roizman

Nescis, mi fili, quantilla rationae mundus regatur.
Axel Gustafsson Oxenstierna (1583–1654)

The search for the etiologic agents of infectious diseases has culminated in the discovery of hundreds of viruses. The pathologic effects of virus diseases result from the interplay of several factors: (a) toxic effects of virus gene products on the metabolism of infected cells, (b) reactions of the host to infected cells expressing virus genes, and (c) modifications of host gene expression by structural or functional interactions with the genetic material of the virus. In most instances the symptoms and signs of acute virus diseases can be related directly to the destruction of cells by the infecting virus. The keys to understanding how viruses infect cells, express their gene functions, multiply, and ultimately alter the cells they infect form a set of concepts and definitions. This chapter summarizes these concepts and definitions and provides a comparison of the various strategies of virus multiplication used by viruses that infect humans. Details of the individual pathways of infection and replication, as well as the specific references, are listed in the chapters dealing with the individual virus families.

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HOST RANGE, SUSCEPTIBILITY, AND PERMISSIVITY

For a virus to multiply, it must first infect a cell. The *host range* of a virus defines both the kinds of tissue cells and the animal species that it can infect and in which it can multiply. The host ranges of different viruses vary considerably; one virus may have a wide host range, whereas the host range of another may be a single cell type of a specific animal species. *Susceptibility* defines the capacity of a cell or animal to become infected. Determinants of host range and susceptibility are discussed in the next section. It should be noted at the outset that, when an individual becomes exposed to a virus with a human host range, cells that become immediately infected are the susceptible cells at the *portal of entry*. However, infection of these cells may not be sufficient to cause clinically demonstrable disease. All too frequently the disease is the consequence of infection of *target* cells (e.g., central nervous system) by virus produced in the susceptible cells at the portal of entry. In many instances (e.g., respiratory infections, genital herpes simplex infections), the target cells are at the portal of entry.

At the onset of infection, the virus introduces into the cell its genetic material—RNA or DNA—accom-

panied in many instances by essential proteins. The sizes, compositions, and gene organizations of viral genomes vary enormously. Viruses appear to have evolved by several routes, and no single pattern of replication has prevailed. In consequence, two concepts must be stressed here.

First, the ability of a virus to multiply and the fate of an infected cell hinge on the synthesis and function of virus gene products—the proteins. Nowhere is the correlation between structure and function—between the composition and arrangement of genetic material and the mechanism of expression of genes—more readily apparent than in viruses. The diversity of mechanisms by which viruses ensure that their proteins are made is reflected in, but alas (!) cannot always be deduced from, their genomic structure.

Second, although viruses differ considerably in the number of genes they contain, all viruses can be said to encode three sets of functions which are expressed by the proteins they specify. Viral proteins (a) ensure the replication of viral genomes, (b) package the genome into virus particles (virions), and (c) alter the structure and/or function of the infected cell.

The strategies employed by viruses to ensure the execution of these functions vary. In a few instances (e.g., papovaviruses, papillomaviruses), viral proteins merely assist host enzymes that replicate the virus genome. In most instances (e.g., picornaviruses, reoviruses, herpesviruses), it is the virus proteins that replicate the virus genome, but even the most self-dependent virus utilizes at least some host proteins in this process. In all instances, it is viral proteins that package the genome into virions even though host proteins and polyamines may complex with viral genomes (e.g., papovaviruses, herpesviruses) before or during the biogenesis of the viral particle. The effects of viral multiplication may range from cell death to subtle, but potentially very significant, changes in function and in antigenic specificity of the infected cell.

Our knowledge concerning reproductive cycles of viruses stems mainly from analyses of the events occurring in synchronously infected cells in culture; we are only beginning to study viruses which do not grow in cultured cells. The reproductive cycles of all viruses exhibit several common features (Fig. 1). First, shortly after infection and for up to several hours thereafter, only small amounts of parental infectious virus can be detected. This interval is known as the eclipse phase; it signals the fact that the virus genomes have been exposed to host or viral machinery necessary for their expression, but that progeny virus production has not yet increased above background. There follows an interval in which progeny virions accumulate in the cell or in the extracellular environment at exponential rates. This interval is known as the maturation phase. After several hours, cells infected with lytic viruses

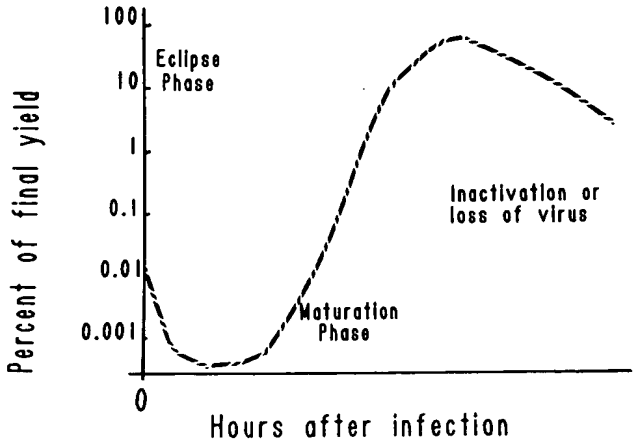

FIG. 1. Reproductive cycle of viruses infecting eukaryotic cells. The time scale varies for different viruses; it may range from 8 hr (e.g., poliovirus) to more than 40 hr (e.g., cytomegalovirus).

cease all metabolic activity and lose their structural integrity. Cells infected with other viruses may continue to synthesize virions indefinitely. The reproductive cycles of viruses range from 6 to 8 hr (picornaviruses) to more than 72 hr (some herpesviruses). Virus yield per cell ranges widely, up to more than 100,000 in the case of poliovirus virions.

Infection of a susceptible cell does not automatically ensure that virus multiplication will ensue and that viral progeny will emerge. This is one of the most important conceptual developments in virology to evolve during the last two decades and should be stressed in some detail. Infection of susceptible cells may be productive, restrictive, abortive, or latent. *Productive* infection occurs in *permissive* cells and is characterized by production of infectious progeny. *Abortive* infections can occur for two reasons. Although a cell may be susceptible to infection, it may be *nonpermissive*, allowing a few, but not all, virus genes to be expressed for reasons that are frequently unknown. Abortive infection may also result from infection of either permissive or nonpermissive cells with *defective* viruses, which lack a full complement of viral genes. Lastly, cells may be only *transiently* permissive, and the consequences are either that the virus persists in the cell until the cell becomes permissive, or that only a few of the cells in a population produce viral progeny at any time. This type of infection has been defined as *restrictive* by some and *restringent* by others. This classification is hardly gratuitous; its significance stems from two observations. The hallmark of *latent infection* is the persistence of viral genomes, but not of infectious virus particles, in transiently nonpermissive cells without the destruction of the infected cell. At the other extreme, persistence of competent or de-

fective viruses which express functions that alter the host genotype without destroying the infected cells may so alter the expression of the host genes as to transform the cell from normal to malignant.

INITIATION OF INFECTION

To infect a cell, the virion must attach to the cell surface, penetrate the cell, and become sufficiently uncoated to make its genome accessible to viral or host machinery for transcription or translation.

Attachment

Attachment constitutes specific binding of a virion protein (the antireceptor) to a constituent of the cell surface (the receptor). The classic example of an antireceptor is the hemagglutinin of influenza virus (orthomyxovirus). The antireceptors are distributed throughout the surfaces of viruses infecting human and animal cells. Complex viruses such as vaccinia (a poxvirus) and herpes simplex virus (a herpesvirus) may have more than one species of antireceptor molecule. Furthermore, antireceptor molecules may have several domains, each of which may react with a different receptor. Mutations in the genes specifying antireceptors may cause loss of the capacity to interact with certain receptors.

The cellular receptors identified so far are largely glycoproteins. Attachment requires ions in the environment in sufficient concentration to reduce electrostatic repulsion, but it is largely temperature and energy independent. The susceptibility of a cell is limited by the availability of appropriate receptors, and not all cells in an otherwise susceptible organism express receptors. Human kidney cells lack receptors for poliovirus when they reside in the organ, but receptors appear when these cells are propagated in cell culture. Susceptibility should not be confused with permissiveness; thus, chick cells are not susceptible to poliovirus inasmuch as they lack receptors for attachment of virions. However, they are fully permissive because they produce virus following transfection with the intact RNA extracted from poliovirus particles.

Attachment of viruses to cells in many instances leads to irreversible changes in the structure of the virion. In some instances, however, when penetration does not ensue, the virus can detach and readsorb to a different cell. Among the viruses in the latter category are orthomyxoviruses and paramyxoviruses, which carry a neuraminidase on their surfaces. These viruses can elute from their receptors by cleaving neuraminic acid from the polysaccharide chains of the receptors.

Penetration

Penetration is an energy-dependent step. It occurs almost instantaneously after attachment and involves one of three mechanisms: (a) translocation of the entire virus across the plasma membrane, (b) endocytosis of the virus particle resulting in accumulation of virus particles inside cytoplasmic vacuoles, and (c) fusion of the cellular membrane with the virion envelope. Nonenveloped viruses penetrate by the first two mechanisms. It is known, for example, that in the course of adsorption of the poliovirus particle to the cell, the capsid becomes modified and loses its structural integrity as the RNA–protein complex is translocated into the cytoplasm. Paramyxoviruses and herpesviruses are examples of viruses that penetrate as a consequence of fusion of their envelopes with the plasma membrane, whereas orthomyxoviruses fuse with cytoplasmic membranes after endocytosis. In these instances the envelope of the virus remains in the plasma membrane, whereas the internal constituents spill into the cytoplasm. Fusion of viral envelopes with the plasma membrane requires the interaction of viral "fusion" proteins in the envelope of the virus with specific protein constituents of the cellular membrane.

Uncoating

Uncoating is a general term applied to the events that occur after penetration, setting the stage for the viral genome to express its functions. For many viruses (orthomyxoviruses, paramyxoviruses, picornaviruses), divestiture of the protective envelope or capsid takes place upon entry into the infected cells. In cells infected with herpesviruses and most likely also with papovaviruses and adenoviruses, the capsid is transported along the cytoplasmic cytoskeleton from the site of entry to nuclear pores. At the nuclear pores, a virus-specific function, triggered most likely by cellular factors at that site, releases the viral DNA or a DNA–protein complex into the nucleus. The empty shell ultimately disintegrates. In cells infected with reoviruses, only portions of the capsid are removed, and the viral genome expresses all its functions even though it is never fully released from the capsid. The poxvirus genome is uncoated in two stages: whereas in the first stage the outer covering is removed by host enzymes, the release of viral DNA from the core appears to require the participation of viral gene products made after infection.

STRATEGIES OF VIRAL MULTIPLICATION

Requirements and Constraints

In the course of their evolution, viruses have developed several strategies to deal with (a) encoding and

organization of viral genes, (b) expression of viral genes, (c) the replication of viral genomes, and (d) assembly and maturation of viral progeny. Before we consider each of these in some detail, it is worth reiterating that the synthesis of viral proteins by the host protein-synthesizing machinery is the key event in viral replication. Irrespective of the size, composition, and organization of its genome, the virus must present to the eukaryotic cell protein-synthesizing machinery a messenger RNA that the cell can recognize as such and translate. In this regard, the cell imposes three constraints on viruses. First, the cell synthesizes its own mRNA in the nucleus by transcription of its DNA followed by posttranscriptional processing of the transcript. The cell therefore lacks (a) the enzymes necessary to synthesize mRNA off a viral RNA genome either in the nucleus or in the cytoplasm and (b) enzymes capable of transcribing viral DNAs in the cytoplasm. The consequence of this constraint is that only viruses whose genomes consist of DNA, contain appropriate *cis*-acting signals, and which reach the nucleus can take advantage of cell transcriptases to synthesize their mRNA. All other viruses had to develop their own enzymes to generate mRNA. The second constraint is that eukaryotic cell protein-synthesizing machinery is equipped to translate only monocistronic messages, inasmuch as it does not usually recognize internal initiation sites within mRNAs. The consequences of this constraint are that viruses must synthesize either a separate mRNA for each gene (functionally monocistronic messages) or an mRNA encompassing several genes and specifying a large precursor "polyprotein" which is then cleaved into individual proteins. Lastly, in the infected cell, the expression of viral genomes is in competition with that of the myriad of cellular genes. To attain abundant amounts of their proteins, viruses have evolved strategies that either conferred competitive advantage to viral mRNAs or abolished the synthesis or translation of cellular mRNAs.

Structure and Organization of Viral Genomes

Viral genes are encoded in either *RNA* or *DNA* genomes. These genomes can be either *single* or *double* stranded. In addition, they can be *monopartite*, that is, all virus genes contained in a single chromosome, or *multipartite*, that is, virus genes are distributed among several chromosomes which together constitute the virus genome. To avoid confusion, we designate as "genomic" only the nucleic acid found in virions.

Among the RNA viruses, reovirus is the representative of the best-known family that contains a double-strand genome; moreover, this genome is multipartite, consisting of 10 segments or chromosomes. The genomes of single-strand RNA viruses can be either monopartite (picornaviruses, togaviruses, paramyxoviruses, rhabdoviruses, coronaviruses, and retroviruses) or multipartite (orthomyxoviruses, arenaviruses, and bunyaviruses). All RNA genomes are linear molecules. Some, for example, picornavirus genomes, contain a covalently linked polypeptide or an amino acid at the 5′ end of the RNA.

All known DNA viruses infecting vertebrate hosts contain a monopartite genome. Except for parvovirus genomes, all are fully or at least partially double stranded. Individual parvovirus virions contain linear single-strand DNA; in some genera (e.g., adeno-associated virus), both complementary strands of the DNA are packaged but in different particles. Papovavirus DNA is packaged in the form of a closed circular molecule, whereas the DNAs of herpesviruses, adenoviruses, and poxviruses are linear. Herpesvirus DNAs exhibit a single 3′ nucleotide extension at both termini in packaged form but circularize immediately after infection. Adenoviruses contain a protein covalently linked to the terminus of one DNA strand, and the viral genome retains its linear configuration throughout infection. The DNA of hepatitis B virus is a circular double-strand molecule in which there are single-strand gaps—one large and one small—in different regions of each strand. In the poxvirus genomes, the 3′ terminus of one strand is covalently linked to the 5′ terminus of the other strand, forming a continuous loop.

Expression and Replication of Viral Genomes

It is convenient to discuss the RNA viruses first and to focus primarily on the function of the genomic RNA.

Single-Strand RNA Viruses

The single-strand RNA viruses form three groups. Picornaviruses and togaviruses are examples of the first group. These genomes have two functions (Figs. 2 and 3), the first of which is to serve as a messenger RNA. By convention, viruses whose genomes can and do serve as messengers are known as *plus-* (+) strand viruses. Following entry into the cell, picornavirus RNA binds to ribosomes and is translated in its entirety (Fig. 2). The product of this translation, the *polyprotein*, is then cleaved. The second function of the genomic RNA is to serve as a *template* for synthesis of a complementary *minus-* (−) strand RNA by a polymerase derived from cleavage of the polyprotein. The (−) strand RNA then serves in turn as a template to make more (+) strands. The progeny (+) strands can then serve as (a) mRNA, (b) templates to make more

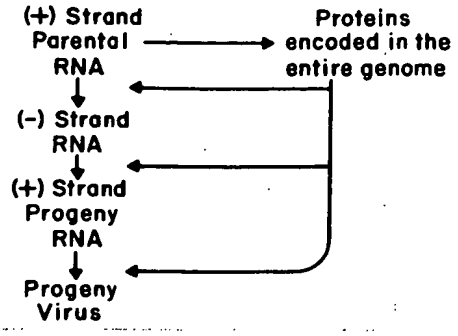

FIG. 2. Flow of events during the replication of picornaviruses.

(−) strands, and (c) constituents of progeny virus particles.

Togaviruses and some of the other (+) strand viruses differ in one respect from picornaviruses (Fig. 3). Specifically, only a portion of the genomic RNA is available for translation in the first round of protein synthesis. The probable function of the resulting products is to transcribe the genomic RNA. A (−) strand is then synthesized, and this RNA in turn serves as a template for two size classes of (+) RNA molecules. In cells infected with togaviruses, the first class is a small mRNA encompassing the region of the genomic RNA not translated in the first round. The resulting polyprotein is cleaved into proteins whose main function is to serve as structural components of the virions. The second class of (+) RNA consists of full-size strands that are packaged into virions. Several mRNA species are made in cells infected with coronaviruses.

Central to the replication of (+) strand viruses is the capability of the genomic RNA to serve as mRNA after infection. The consequences are twofold. First, enzymes responsible for the replication of the genome are made after infection and need not be brought into the infected cell by the virion. That is why naked RNA extracted from virions of (+) strand viruses is infec-

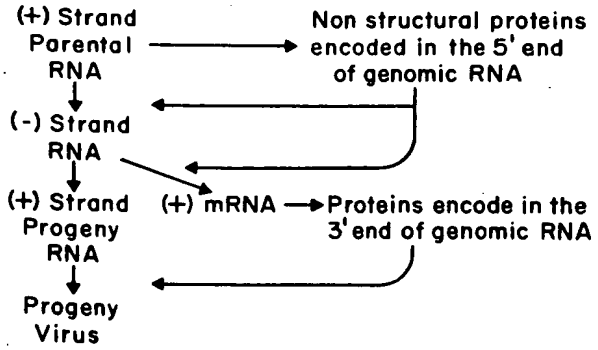

FIG. 3. Flow of events during the replication of togaviruses.

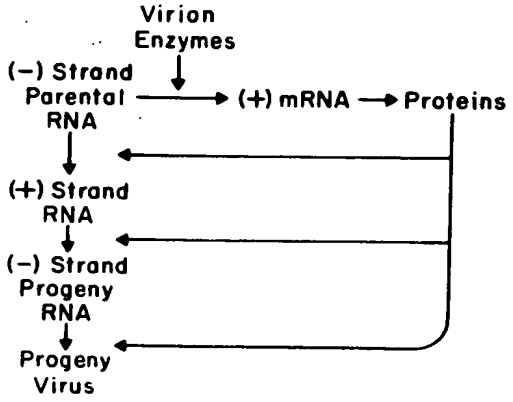

FIG. 4. Flow of events during the replication of orthomyxoviruses and paramyxoviruses.

tious. Second, because all (+) strand genomes are monopartite, and therefore have all their genes linked in a single chromosome, the initial products of translation of both genomic RNA and mRNA species are necessarily a single protein. The translation products of picornaviruses and togaviruses must then be cleaved to yield the individual proteins found in the virion or the infected cell.

Orthomyxoviruses, paramyxoviruses, bunyaviruses, arenaviruses, and rhabdoviruses (Fig. 4) comprise the second set of single-strand RNA viruses defined as the (−) strand viruses. Characteristically, their genomic RNAs must serve two template functions, first for transcription and then for replication. Because their genome must be transcribed to make mRNA, and the cell lacks the appropriate enzymes, all (−) strand viruses package in the virion a transcriptase along with the viral genome. The transcription of the viral genome is the first event after entry of the virus into cells; the process yields functionally monocistronic mRNAs [(+) strands], each specifying a single protein. Replication begins under the direction of newly synthesized viral proteins; a full-length (+) strand is made and serves as a template for the synthesis of (−) strand genomic RNAs (Fig. 4).

Central to the replication of the (−) strand viruses is that the genomic RNAs function alternatively as templates for transcription and replication. The consequences are threefold. First, the virus must bring into the infected cell the transcriptase to make its mRNAs. Second, it follows that naked RNA extracted from virions is not infectious. Third, the mRNAs produced are gene unit length: they specify a single polypeptide. However, selective (but not arbitrary) observance of RNA splicing signals may result in multiple mRNAs, each specifying a different protein being transcribed from the same region of genomic RNA. Consequently, the (+) transcript which functions as mRNA is different from the (+) strand RNA which

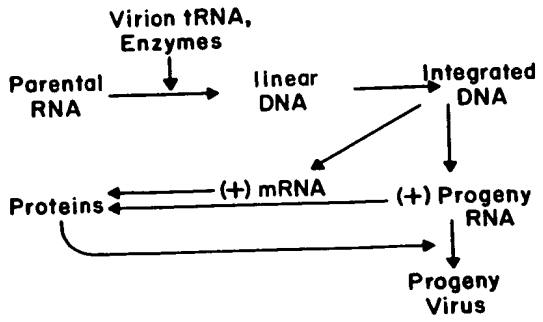

FIG. 5. Flow of events during the replication of retroviruses.

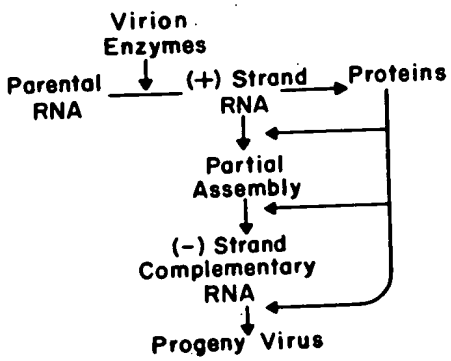

FIG. 6. Flow of events during the replication of reoviruses.

serves as the template for progeny virus, even though both are synthesized on the genomic RNA. The advantages accrued by the transcription of multiple mRNAs from the same region through splicing of the RNA are obvious. Monocistronic mRNA is advantageous because the virus can control the abundance of the individual proteins; they need not be made in equimolar amounts.

The classification of single-strand RNA viruses into (+) and (−) groups does contain an exception: A few members of the (−) strand RNA, multipartite bunyavirus family are *ambisense*; that is, both the genomic and complementary (+) strand of a chromosome can act as mRNA.

Retroviruses comprise the third group of single-strand RNA viruses (Fig. 5). Characteristically, retrovirus genomes are monopartite but diploid, and the two strands are either partially hydrogen bonded to another macromolecule or base paired in a fashion as yet unknown. The sole function of the genomic RNA is to serve as a template for the synthesis of viral DNA. Inasmuch as eukaryotic cells lack enzymes competent to perform this function, the virion contains, in addition to the genome, an RNA-dependent DNA polymerase (reverse transcriptase) as well as a mixture of host transfer RNAs, one of which serves as a primer. The key steps in the reproductive cycle are (a) binding of the tRNA–reverse transcriptase complex to the genomic RNA, (b) synthesis of a DNA copy complementary to the RNA across the two ends of the RNA molecule in such a fashion as to produce a single-strand DNA molecule hydrogen bonded to the linear genomic RNA, (c) digestion of genomic RNA by a nuclease which attacks only RNA in DNA–RNA hybrids (ribonuclease H, also packaged in the virion), and (d) synthesis of the complementary strand of the viral DNA. The linear double-strand DNA translocated into the nucleus integrates into the host genome. Virus gene expression may not follow immediately. All retroviruses contain *cis*-acting sites for cellular *trans*-acting factors (that may be tissue specific) and for the host RNA polymerase. The products of transcription are genome-length RNA molecules and shorter, gene-cluster-length mRNAs that are translated to yield polyproteins. The polyproteins are then cleaved to yield the individual viral proteins. Only the genome-length transcript is packaged into virions. The complexity of regulation varies among retroviruses: detailed analyses of lentiviruses have revealed several virally encoded *trans*-acting factors that regulate the order of expression and the abundance of viral gene products.

Double-Strand RNA Viruses

The double-strand, multipartite reovirus genome is transcribed within the partially opened capsid by a polymerase packaged into the virion, and the 10 mRNA [(+) strand] species are extruded through the exposed vertices of the capsid (Fig. 6). The mRNA molecules have two functions. First, they are translated as monocistronic messages to yield the viral proteins. Second, one RNA of each of the 10 species assembles within a precursor particle in which it serves as a template for synthesis of the complementary strand, yielding double-strand genome segments.

DNA Virus Genomes

The DNA viruses can be split into four groups. Papovavirus, papillomavirus, adenovirus, and herpesvirus genomes are transcribed and replicated in the nucleus and therefore can utilize the transcriptional enzymes of the host for generation of mRNA. Consistent with this, DNAs of the viruses are infectious. The transcriptional program consists of at least two cycles of transcription for papovaviruses and at least three for herpesviruses (Fig. 7) and adenoviruses. In each instance, the structural or virion polypeptides are made largely from mRNA generated from the last cycle of transcription. In the case of nuclear DNA viruses,

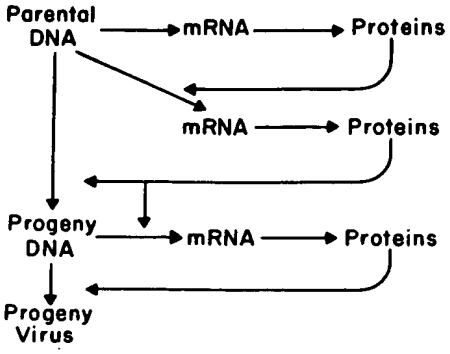

FIG. 7. Flow of events during the replication of herpesviruses (herpes simplex viruses).

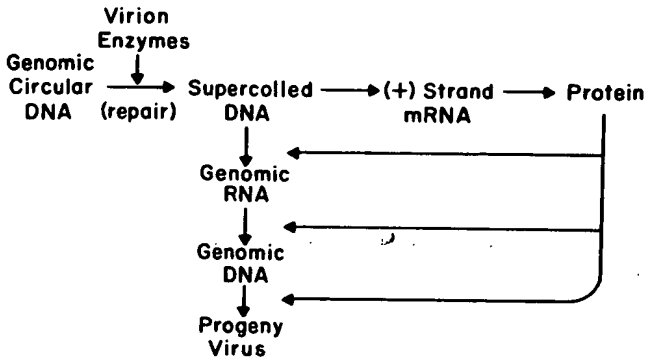

FIG. 8. Flow of events during the replication of hepatitis B virus.

two sets of *cis*-acting sites are embedded in the domains of viral genes. One set enables the binding of cellular transcriptional factors and *trans*-acting factors for initiation of transcription and enhanced expression of these genes. The second set enables the interaction of these genes with viral regulatory proteins which may regulate transcription both positively and negatively. In the case of some herpesviruses, a virion protein introduced into the cell during infection *trans*-activates the first set of viral genes to be expressed by acting in concert with a host protein that binds to its *cis* sites in the viral genome.

Although it is convenient to lump the nuclear DNA viruses together, significant differences do exist in their replication strategies. Papovaviruses encode a single protein that binds in close proximity to the origin of viral DNA synthesis, stimulates the cellular polymerase complex to replicate the viral DNA, and acts as a helicase. Adenoviruses encode a DNA polymerase but depend on the host cells for many of the other functions involved in the synthesis of their DNA. At the other extreme are the herpesviruses. Herpes simplex viruses encode numerous proteins involved in the pathway of synthesis of DNA. The list includes seven proteins that are both necessary and sufficient for viral origin-dependent synthesis of DNA in the environment of the uninfected cell. In the case of other herpesviruses, the genome encodes additional proteins that maintain the viral DNA in an episomal state and stimulate the cellular DNA replication proteins to amplify the DNA to an appropriate copy number.

The poxviruses constitute the second group. Although poxvirus DNAs have been detected in the nucleus, at least the initial transcriptional events and most of the other events in the reproductive cycle appear to take place in the cytoplasm. As could be predicted from their localization, poxviruses have evolved all the factors necessary for transcription and replication of their genome. Furthermore, because host transcriptional factors are not involved, the *cis*-acting sites for the synthesis and processing of the mRNA have diverged from those of the host. The initial transcription occurs in the core of the virion; the protein products of these transcripts function to release the viral genome from the core. Many questions concerning the reproductive cycle of these viruses remain unresolved.

Parvoviruses constitute the third group. The parvoviruses that infect humans form two groups. The members of one are capable of independent replication. The only known member of the second, the adeno-associated virus, is a "defective" virus and requires adenoviruses or herpes simplex viruses as helper viruses for its multiplication. Multiplication requires the synthesis of a DNA strand complementary to the single-strand genomic DNA in the nucleus and transcription of the genome.

Hepatitis B virus exemplifies the fourth group (Fig. 8). The gaps in the DNA of this virus are repaired first, and its genome is converted into a closed circular molecule by a DNA polymerase packaged in the virion. The genome is then transcribed into two classes of RNA molecules, that is, mRNA specifying proteins and a full-length RNA that serves as a template for the synthesis of genomic DNA by a virally encoded reverse transcriptase.

Assembly, Maturation, and Egress of Viruses from Infected Cells

Viruses have evolved three fundamental strategies for their assembly, maturation, and egress from the infected cells. The first, exemplified by picornaviruses, reoviruses, papovaviruses, parvoviruses, adenoviruses, and poxviruses, involves intracellular assembly and maturation. In the case of picornaviruses, 60 copies each of the virion proteins designated VP0, VP1, and VP3 assemble in the cytoplasm into a procapsid. Viral RNA then wraps around the procapsid,

and, in the process, VP0 is cleaved to yield two polypeptides, VP2 and VP4. The cleavage probably causes rearrangement of the capsid into a thermodynamically stable structure in which the RNA is shielded from access by nucleases. Poxviruses and reoviruses also assemble in the cytoplasm. In contrast, adenoviruses, papovaviruses, and parvoviruses assemble in the nucleus. As a rule, all nonenveloped viruses that assemble and acquire infectivity inside the cell depend largely, but not entirely, on the disintegration of the infected cell for their egress. Accumulated evidence implicates the structural proteins of these viruses in the inhibition of host macromolecular metabolism and in the ultimate disintegration of the infected cell.

The second strategy is employed by enveloped viruses exemplified by all (−) strand RNA viruses, togaviruses, and retroviruses. For the viruses in this group, the last step in virion assembly is linked with its egress from the infected cell. Specifically, some of the virus proteins become inserted into both the inner and outer surface of the plasma membrane or other cytoplasmic membranes; those projecting into the extracellular fluid or into the cisternae of the endoplasmic reticulum are glycosylated. The membrane proteins aggregate into patches displacing host membrane proteins. Viral nucleocapsids bind to specific virus-specified proteins lining the cytoplasmic side of these patches or to cytoplasmic domains of viral glycoproteins (togaviruses) and become wrapped up by the patch. In the process, the nascent virion is "extruded" or "buds" into the extracellular environment. In some instances (e.g., orthomyxoviruses and paramyxoviruses), cleavage and rearrangement of one species of surface protein occur during or after extrusion and impart to the newly formed virion the capability of infecting cells. Virus assembly and maturation by extrusion from the cell surface provide a more efficient mechanism of egress inasmuch as it does not depend on disintegration of the infected cell. Indeed, viruses that mature and egress in this fashion vary considerably in their effects on host-cell metabolism and integrity. They range from highly cytolytic (togaviruses, paramyxoviruses, rhabdoviruses) to virtually noncytolytic (retroviruses). However, by virtue of the insertion of the viral glycoproteins into the cell surface, these viruses impart to the cell a new antigenic specificity, and the infected cell can and does become a target for the immune mechanisms of the host.

Lastly, the herpesvirus nucleocapsid is assembled in the nucleus. Unlike other enveloped viruses, the envelopment and maturation occur at the inner lamella of the nuclear membrane. The enveloped virus accumulates in the space between the inner and outer lamellae of the nuclear membrane, in the cisternae of the cytoplasmic reticulum, and in vesicles carrying the virus to the cell surface. Thus, the enveloped virus is uniquely shielded from contact with the cytoplasm. Herpesviruses are cytolytic and invariably destroy the cells in which they multiply. Like other enveloped viruses, herpesviruses impart to the infected cell new antigenic specificities.

CONCLUSIONS

Viruses exhibit a remarkable array of strategies for the expression of their genes and for the replication of their genomes. Knowledge of the biology of viruses and, specifically, the host range, target cell, portal of entry, and strategies of gene expression and of replication of the viral nucleic acids is the key to the development of rational and effective methodologies for prevention and treatment of viral diseases. The discovery of new viruses, particularly those whose host range comprises a small subset of highly differentiated cells (e.g., human immunodeficiency viruses, human herpesvirus 6), suggests that more viruses with novel strategies of gene expression and amplification of their nucleic acids remain to be discovered.

The focus of the chapters on the individual virus groups is the mechanisms by which the various strategies of known viruses are implemented.

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CHAPTER 65

Herpes Simplex Viruses and Their Replication

Bernard Roizman and Amy E. Sears

> On fait la science avec des faits, comme on fait une maison avec des pierres; mais une accumulation de faits n'est pas plus une science qu'un tas de pierres n'est une maison.
>
> HENRI POINCARE

Herpes simplex viruses (HSVs) were the first of the human herpesviruses to be discovered and are among the most intensively investigated of all viruses. Their attractions are their biologic properties and, in particular, their ability to cause a variety of infections, to remain latent in their host for life, and to be reactivated to cause lesions at or near the site of initial infection. They serve as models and tools for the study of translocation of proteins, synaptic connections in the nervous system, membrane structure, gene regulation, and a myriad of other biological problems, both general to viruses and specific to HSV. For years, their size and complexity served as a formidable obstacle to intensive research. More than 40 years passed from the time of their isolation until Schneweiss (444) demonstrated that there were, in fact, two serotypes, HSV-1 and HSV-2, whose formal designations under ICTV

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rules are now human herpesviruses 1 and 2 (416). Not until 1961 were plaque assays published (420), and only much later were the genome sizes and the extent of homology between these two viruses reported. This chapter recounts well-established facts; however, its main emphasis is on burning issues, the problems whose time has come.

Virology conserves two myths. The first is that research on a virus reaches its peak when the number of investigators approaches the number of nucleotides in its genome. This formula calls for 152,000+ investigators, one for each base pair (222,294). In orders of magnitude, we are close but not yet there. There are times when we think almost that many bodies will be needed to unravel all the mysteries of these viruses.

The second myth is that virologists repeat the same experiment over and over again. As in all myths, there may be a grain of truth here. In wading through the mass of articles published in the past few years, it was instructive to see how many times the same phenomenon was published or rediscovered time and time again. This apparently is not only a reflection of George Santayana's injunction that "Those who cannot remember the past are condemned to repeat it" but is also because only the last reported experiment is remembered, correct or not. We have taken pains to correct the record.

VIRION STRUCTURE

As with all herpes virions, the HSV virion consists of four elements: (i) an electron-opaque core, (ii) an icosadeltahedral capsid surrounding the core, (iii) an amorphous tegument surrounding the capsid, and (iv) an outer envelope exhibiting spikes on its surface.

Virion Polypeptides

Studies on purified HSV-1 virions indicated that they contain approximately, but probably not less than, 33 proteins designated as virion polypeptides (VPs) and given serial numbers (170,478). All of the virion proteins were made after infection, and no host protein could be detected in purified preparations. Of the approximately 33 proteins, eight are on the surface of the virion and are glycosylated. These glycoproteins are gB (VP7 and VP8.5), gC (VP8), gD (VP17 and VP18), gE (VP12.3 and VP12.6), gG, gH and gI. Another gH small glycoprotein, which will be given the designation gJ, predicted by DNA sequence analyses, has recently been demonstrated by N. Frenkel and associates (N. Frenkel, *personal communication*). Some of the surface glycoproteins have been shown to be components of the envelope spikes (482).

Gibson and Roizman (148,150) described three kinds of capsids, namely, those that lack DNA and were never enveloped (type A), those that contain DNA and were never enveloped (type B), and those that contain DNA and were obtained by de-enveloping intact virions (type C). The empty capsids consist of five proteins, namely, VP5, VP19C, VP23, VP24, and a smaller (12,000 molecular weight) protein described subsequently (64). VP5 was estimated to be present in ratios of 850–1,000 per virion [i.e., approximately six per hexameric capsomere (170,417,511)], but recent studies (445) suggest that VP5 is a component of both pentameric and hexameric capsomeres. VP19C and VP5 appear to be linked by disulfide bond (550) and are present in approximately similar ratios per virion (170). Braun et al. (32) showed that VP19C, identified as the infected cell protein (ICP) 32, bound DNA and was probably involved in anchoring the viral DNA in the capsid. Recent studies by Sherman and Bachenheimer (462) suggest that the type A capsids are not in the pathway of virion maturation and may be a decay product.

Type B capsids differ from the A type in that they contain two additional proteins, namely, VP21 and VP22a. Type C capsids were reported to contain a smaller protein VP22 but not VP22a (148,150). VP22 and VP22a have similar characteristics (148,150) and were thought to be related constituents of the ICP35 family of proteins present on the surface of the capsid (34) and required for encapsidation of viral DNA (377,399,462). Gibson and Roizman (148) suggested that VP21 is an internal capsid protein.

Depending on the procedure for stripping the envelope, the type C capsids may contain variable amounts of tegument proteins. The type C capsids lack protein VP22a but contain protein VP22. Gibson and Roizman (148,150) concluded that VP22 is processed from VP22a by proteolytic cleavage, since the proteins appear to have similar characteristics. Recent studies by Sherman and Bachenheimer (462) and Rixon et al. (399) suggest that the VP22 found in the type C capsids may not be related to VP22a.

The space between the undersurface of the envelope and the surface of the capsid was designated as the *tegument* (417); it contains the rest of the virion proteins. The most notable of the proteins associated with the underside of the envelope and the capsid are the α-*trans*-inducing factor (αTIF; ICP25; VP16), the virion host shut-off (VHS) protein, and a very large protein (VP1) associated with a complex that binds to the terminal (*a*) sequence of the viral genome. Extensive discussion of the various types of capsids and virions was published by Roizman and Furlong (417). Schrag et al. (445) have reported an elegant model of the HSV-1 capsid.

Viral DNA

Like other herpesvirus DNAs, HSV DNA is linear and double-stranded (23,222,361). In the virion, HSV DNA is packgaged in the form of a toroid (141). The ends of the genome are probably held together or are in close proximity inasmuch as the DNA circularizes rapidly in the absence of protein synthesis after it enters the nuclei of infected cells (362). DNA extracted from virions contains nicks and gaps (27,135,540).

The HSV genome is approximately 150 kilobase pairs, with a G+C content of 68% (HSV-1) or 69% (HSV-2) (23,222,294). It consists of two covalently linked components, designated as L (long) and S (short) (Fig. 1). Each component consists of unique sequences bracketed by inverted repeats (460,514). The repeats of the L component are designated *ab* and *a'b'*, while those of the S component are designated *a'c'* and *ca* (514) (Fig. 1). The number of *a* sequence repeats at the L–S junction and at the L terminus is variable; the HSV genome can then be represented as

$$a_L a_n b—U_L—b'a'_m c'—U_s—ca_s$$

where a_L and a_s are terminal sequences with unique properties described below, and a_n and a_m are terminal *a* sequences directly repeated zero or more times (n) or present in one to many copies (m) (87,270,411,412, 504,514,519). The structure of the *a* sequence is highly conserved but consists of a variable number of repeat elements. In the HSV-1(F) strain, the *a* sequence consists of a 20-base-pair direct repeat (DR1), a 65-base-pair unique sequence (U_b), a 12-base-pair direct repeat (DR2) present in 19–23 copies per *a* sequence, a 37-base-pair direct repeat (DR4) present in two to three copies, a 58-base-pair unique sequence (U_c), and a final copy of DR1 (318,320). The size of the *a* sequence varies from strain to strain, reflecting in part the number of copies of DR2 and DR4. The structure of the *a* sequence can be represented as

$$DR1—U_b—DR2_n—DR4_m—U_c—DR1$$

with adjacent *a* sequences sharing the intervening DR1. Linear virion DNA contains asymmetric ends, with the terminal *a* sequence of the L component (a_L) ending with 18 base pairs and one 3′ nucleotide extension, and the terminal *a* sequence of the S component (a_s) ending with a DR1 containing only 1 base pair and one 3′ overhanging nucleotide (320).

The L and S components of HSV can invert relative to one another, yielding four linear isomers (Fig. 1) (92,169). The isomers have been designated as P (prototype), I_L (inversion of the L component), I_s (inversion of the S component), and I_{SL} (inversion of both S and L components) (169,323,324).

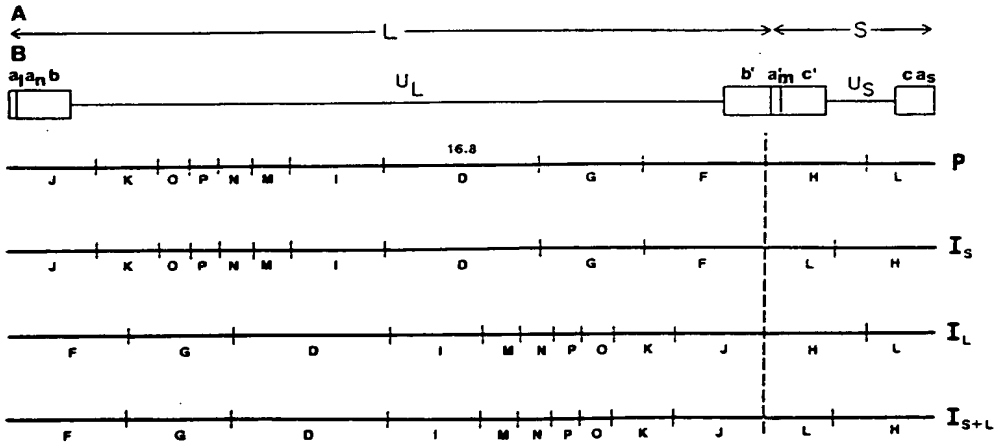

FIG. 1. Schematic representation of the arrangement of DNA sequences in the HSV genome. **A:** The domains of the L and S components are denoted by the arrows. The second line shows the unique sequences (*thin lines*) flanked by the inverted repeats (*boxes*). The letters above the second line designate the following: the terminal *a* sequence of the L component (a_L); a variable (n) number of additional *a* sequences; the *b* sequence; the unique sequence of the L component (U_L); the repetition of the *b* and of a variable (m) number of *a* sequences (a_m); the inverted *c* sequence (c'); the unique sequence of the S component (U_s); and, finally, the terminal *a* sequence (a_s) of the S component. **B:** The Bg1II restriction endonuclease map of HSV-1(F) strain for the P, I_s, I_L, and I_{SL} isomers of the DNA. Note that because Bg1II does not cleave within the inverted repeat sequences, there are four terminal fragments and four fragments spanning the internal inverted repeats in concentrations of 0.5 and 0.25 M, respectively, relative to the concentration of the viral DNA.

The evidence for the repetition of terminal sequences in inverted orientation was based on electron-microscopic studies of denatured HSV-1 DNA allowed to self-anneal (460). These studies, as well as partial denaturation profiles of HSV DNA, revealed that the terminal repeats are repeated internally and that the repeats of each end differ in size and sequence arrangements (92,514). The demonstration that restriction endonucleases which cleave outside inverted repeats yield four 0.5 M terminal fragments and four 0.25 M L–S component junction fragments (169) supported the conclusion that L and S components can invert relative to each other.

The internal inverted repeat sequences are not essential for growth of the virus in cell culture; mutants from which portions of unique sequences and most of the internal inverted repeats have been deleted have been obtained in all four arrangements of HSV DNA (203,363). The genomes of these mutants do not invert; each is frozen in one arrangement of the L and S components, but all retain their viability in cell culture.

Other Constituents

Polyamines

The search for polyamines in the virion evolved from the observations that HSV capsid assembly requires addition of arginine to the medium (284,426,498) and that the capsid does not contain highly basic proteins that would neutralize viral DNA for proper folding inside the capsid. Highly purified virions contain the polyamines spermidine and spermine in a nearly constant ratio, yielding approximately 70,000 molecules of spermidine and 40,000 molecules of spermine per virion (147,149). The polyamines appear to be tightly bound, and they cannot be exchanged with exogenously added labeled polyamines. Disruption of the envelope with nonionic detergents and urea removed the spermidine but not the spermine. The spermine contained in the virion is sufficient to neutralize approximately 40% of the DNA phosphate (149). Parenthetically, proteins have been noted in association with the toroidal structure (141) in the capsid, and a capsid protein has been reported to bind DNA (32).

The compartmentalization of spermine and spermidine may reflect the distribution of polyamines in the infected cell. It is of interest to note that after infection, the conversion of ornithine to putrescine appears to be blocked, but the synthesis of spermine and spermidine does not appear to be affected (147).

Lipids

It has been assumed that HSV acquires its envelope lipids from the host cells. Little is known of the composition of the lipids in the envelopes. The hypothesis that it is determined by the host was supported by the observation that the buoyant density of the virus was host-cell-dependent on serial passage of HSV-1 alternately in HEp-2 and chick embryo cells (477). Since the envelope is derived from cellular membranes, it has been assumed that the viral envelope and cellular membranes contain similar or identical lipids. Even when analyses of viral lipids were in vogue, little was learned of the lipids in the HSV virion. What little is known has been reviewed in detail elsewhere (417).

HSV Polymorphism

Intertypic Variation

Although the genetic maps of HSV-1 and HSV-2 are largely collinear, they differ in restriction endonuclease cleavage sites and in the apparent sizes of viral proteins. Thus, the initial locations of viral genes on the linear map of HSV genomes were based on analyses of HSV-1 × HSV-2 recombinants and took advantage of (a) the intertypic difference in the sizes of the proteins and (b) the locations of restriction endonuclease cleavage sites (286,323,324,378).

Intratypic Variation

The first evidence of intratypic polymorphism emerged from studies of virion structural proteins and indicated that nonglycosylated proteins vary sufficiently in electrophoretic mobility to be used as strain markers (355). Intratypic variability was also noted by Pereira et al. (356) in their studies on the distribution of epitopes to specific monoclonal antibodies among HSV-1 and HSV-2 isolates. The usefulness of virion proteins as markers for molecular epidemiologic studies was limited by the effort required to purify virions for such analyses.

At the DNA level, differences between HSV-1 strains appear to result from (a) base substitutions which may add or eliminate a restriction endonuclease cleavage site and which may, on occasion, change an amino acid or (b) variability in the number of repeated sequences present in a number of regions of the genome (e.g., γ34.5, US11, etc.) (57,400). The restriction endonuclease cleavage patterns of a given strain are relatively stable, whereas the number of repeats are not (37,168,415,504). Thus, no changes in restriction endonuclease patterns were noted in isolates from the same individual over an interval of 13 years or in genomes of an HSV-1 strain passaged serially numerous times in cell culture. However, restriction endonuclease site polymorphism was readily noted in isolates from epidemiologically unrelated individuals

(164,427). On the basis of these properties, restriction endonuclease site polymorphism was used in several epidemiologic studies of HSV transmission in the human population (37,415,427), and restriction endonuclease analyses of coded virus isolates have been used to trace the spread of infection from patients to hospital personnel (35), from patient to patient (268), and from hospital personnel to patient (4,36).

VIRAL REPLICATION

The General Pattern of Replication

It is convenient to begin this section on viral replication with a bird's-eye view of the major events (Fig. 2).

To initiate infection, the virus must attach to cell receptors. Fusion of the envelope with the plasma membrane rapidly follows the initial attachment. The de-enveloped capsid is then transported to the nuclear pores, where DNA is released into the nucleus.

Transcription, replication of viral DNA, and assembly of new capsids take place in the nucleus (Fig. 3).

Viral DNA is transcribed throughout the reproductive cycle by host RNA polymerase II, but with the participation of viral factors at all stages of infection. The synthesis of viral gene products is tightly regulated (Fig. 4): Viral gene expression is coordinately regulated and sequentially ordered in a cascade fashion, with the approximately 70+ gene products forming at least five groups on the basis of both transcriptional and posttranscriptional regulation.

Several of the gene products are enzymes and DNA-binding proteins involved in viral DNA replication. The bulk of viral DNA is synthesized by a rolling circle mechanism, yielding concatemers that are cleaved into monomers and packaged into capsids.

Assembly occurs in stages: After packaging of DNA into preassembled capsids, the virus matures and acquires infectivity by budding through the inner lamellae of the nuclear membrane (Fig. 5) In fully permissive tissue culture cells, the process takes approximately 18–20 hr.

Initial Stages of Infection

The available information on events preceding the transcription of viral genes is still fragmentary. The

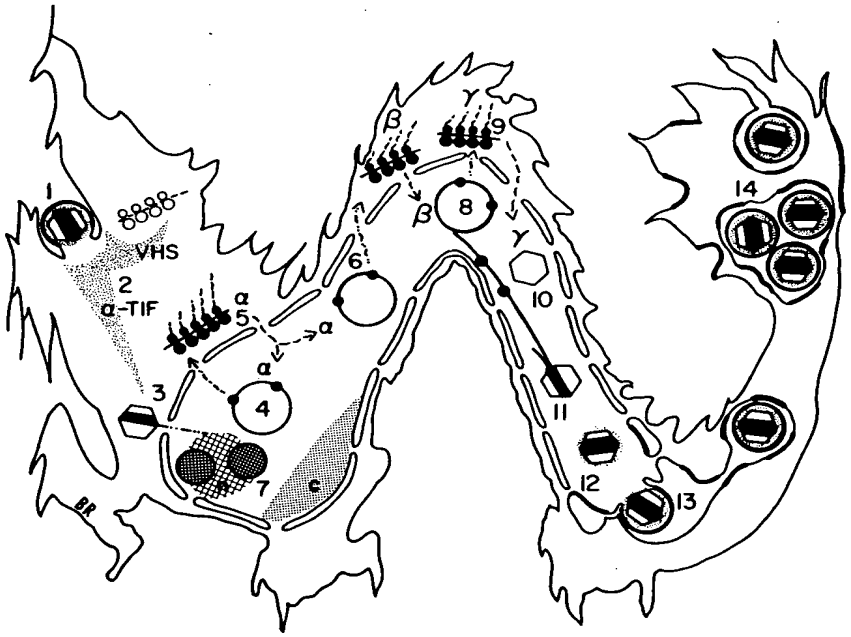

FIG 2. Schematic representation of the replication of herpes simplex viruses in susceptible cells. *1*: The virus initiates infection by the fusion of the viral envelope with the plasma membrane following attachment to the cell surface. *2*: Fusion of the membranes releases two proteins from the virion. VHS shuts off protein synthesis (*broken RNA in open polyribosomes*). αTIF (the α gene *trans*-inducing factor) is transported to the nucleus. *3*: The capsid is transported to the nuclear pore, where viral DNA is released into the nucleus and immediately circularizes. *4*: The transcription of α genes by cellular enzymes is induced by αTIF. *5*: The five α mRNAs are transported into the cytoplasm and translated (*filled polyribosome*); the proteins are transported into the nucleus. *6*: A new round of transcription results in the synthesis of β proteins. *7*: At this stage in the infection, the chromatin (c) is degraded and displaced toward the nuclear membrane, whereas the nucleoli (*round, hatched structures*) become disaggregated. *8*: Viral DNA is replicated by a rolling circle mechanism that yields head-to-tail concatemers of unit-length viral DNA. *9*: A new round of transcription/translation yields the γ proteins consisting primarily of structural proteins of the virus. *10*: The capsid proteins form empty capsids. *11*: Unit-length viral DNA is cleaved from concatemers and packaged into the preformed capsids. *12*: Capsids containing viral DNA acquire a new protein. *13*: Viral glycoproteins and tegument proteins accumulate and form patches in cellular membranes. The capsids containing DNA and the additional protein attach to the underside of the membrane patches containing viral proteins and are enveloped. *14*: The enveloped capsids accumulate in the endoplasmic reticulum and are transported into the extracellular space.

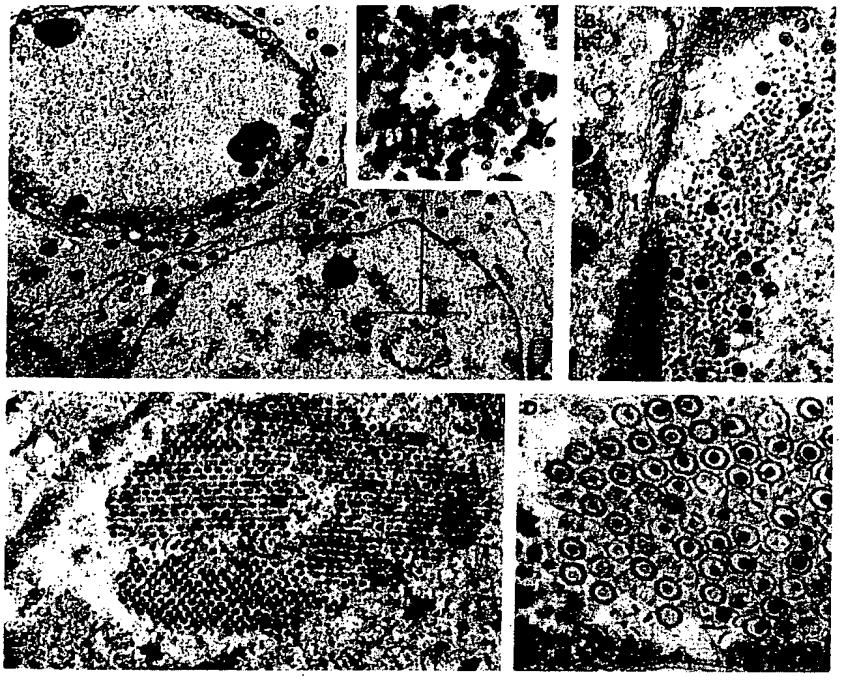

FIG. 3. Electron micrographs of the intracellular events in HSV-1 replication. **A:** Electron-opaque bodies (magnified in insert) showing sites of assembly of capsids, **B:** A region near the edge of the nucleus showing accumulation of chromatin, small particles that appear to be capsid precursors, and capsids. **C:** A paracrystalline array of capsids, both empty and containing DNA, frequently found in nuclei of infected cells. **D:** Capsids in nuclei of infected cells in various stages of packaging of viral DNA. [Electron micrographs have been assembled from refs. 410 and 417 and from J. Schwartz and B. Roizman (*unpublished micrographs*).]

central issue is that two of the initial events—(i) attachment to the cell surface and (ii) fusion of the viral envelope with the plasma membrane—must necessarily involve viral surface proteins. Of the eight HSV glycoproteins, five (gC, gE, gG, gI, and gJ) are dispensable in cell culture, both for entry into cells and for egress from cells (170,273–275,526). As a matter of principle, we do not accept the notion that the virus conserves unnecessary genes or has evolved gratuitous targets for the host immune system. The hypothesis that we raise but do not test is that the five dispensable glycoproteins perform the functions necessary for one or more alternative steps in the initial stages in infection. If that is true, the three essential glycoproteins (gB, gD, and gH) represent the minimal set of surface proteins necessary to sustain and carry out the dominant flow of events. The preferred scenario described in the following sections probably reflects the functions only of this minimal set of surface proteins.

Attachment

HSV-1 and HSV-2 are readily detected on the surface of cells juxtaposed to plasma membranes of cells exposed for a brief interval to infectious virus (Fig. 6). Recent studies by Spear and colleagues (547) indicate that the receptor molecules recognized in one of the initial binding events are heparan sulfate proteoglycans. Consistent with this view, attempts to find cultured cells lacking receptors have not been successful, leaving the species specificity of natural infection by this virus a mystery: other than humans, only chimpanzees are "naturally" infected with this virus (292). Spear and colleagues have demonstrated, by inhibition of attachment of virus by glycoproteins and synthetic peptides, that either gB or gC is required for this step in the process of attachment of HSV to cell surfaces (P. G. Spear, *personal communication*). Whether both glycoproteins see the same domain of heparan sulfate proteoglycans is not yet clear. The attachment of virus to heparan sulfate is the first step in the attachment process.

An apparently similar step in the attachment process is the one that is blocked by the polycations neomycin and polylysine (257,258; G. Campadelli-Fiume, *unpublished data*). Mapping data based on the differences in susceptibility of HSV-1 and HSV-2 to these compounds suggests that this step appears to involve gC. Minson and co-workers (38,99) have recently shown that gH is also required for one of the early steps in viral infection, but this step may involve penetration rather than attachment.

The approach taken in the attachment studies described above is in stark contrast to the many attempts to define the components of the attachment process by analyses of attachment and penetration of viruses exposed to polyclonal or monoclonal antibodies directed against individual proteins. We have set aside the observations that mono- or polyclonal antibodies to each of the major glycoproteins (gB, gC, gD, gE)

may inhibit infection or preclude penetration but not attachment. These catalogues of interesting data may become useful when the precise epitopes and the structures of the surface domains of the glycoproteins become known. In their present form, the data are amenable to many different interpretations.

Penetration into the Infected Cell

Attachment to the cell surface activates a process mediated by viral surface proteins that cause the fusion of the viral envelope and the cell plasma membrane. There is overwhelming acceptance of Morgan et al.'s (322) hypothesis that multiplication results from the entry of virus mediated by fusion of the envelope and plasma membranes rather than from that mediated by phagocytosis. This hypothesis is supported by the observation that penetration by endocytosis results in a nonproductive infection (43). The demonstration that virion envelope Fc receptors [i.e., gE and gI (205)] could be detected on cell surfaces following penetration in the absence of viral gene expression is consistent with this hypothesis (345).

Penetration may be a multistep event involving more than one viral glycoprotein. The cumulative evidence

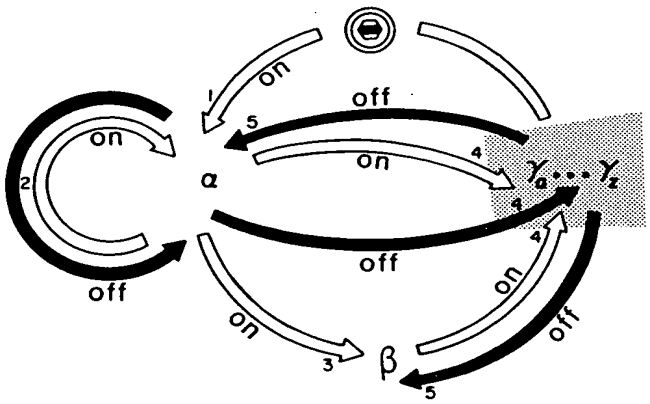

FIG. 4. Schematic representation of the regulation of HSV-1 gene expression. Open and filled arrows represent events in the reproductive cycle which turn gene expression "on" and "off," respectively. *1*: Turning on of α gene transcription by αTIF, a γ protein packaged in the virion. *2*: Autoregulation of α gene expression. *3*: Turning on of β gene transcription. *4*: Turning on of γ gene transcription by α and β gene products through *trans*-activation of γ genes, release of γ genes from repression, and replication of viral DNA. Note that γ genes differ with respect to the stringency of the requirement for DNA synthesis. The heterogeneity is shown as a continuum in which inhibitors of viral DNA synthesis are shown to have minimal effect on γ_a gene expression but totally preclude the expression of γ_z genes. *5*: Turn off of α and β gene expression by the products of γ genes late in infection.

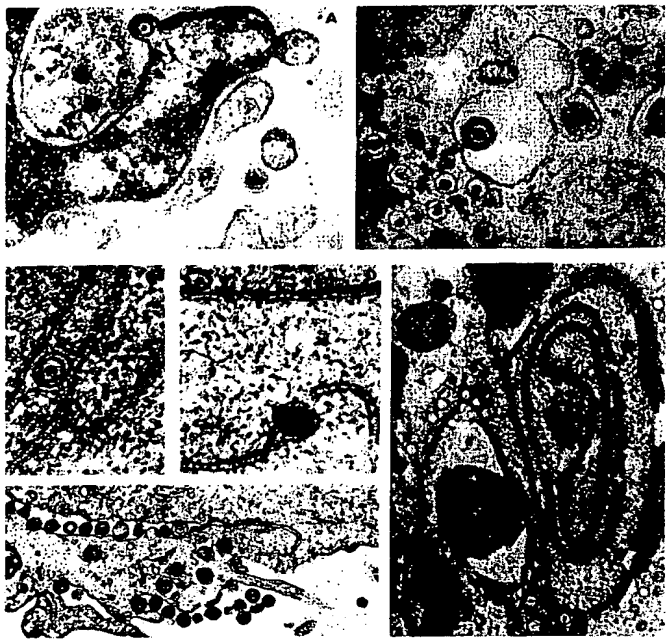

FIG. 5. Electron micrographs of the envelopment and egress of virus from infected cells. **A:** Envelopment of virus from a protrusion of the nucleus. Note that the nucleus contains marginated chromatin. The inner lamellae of the nuclear membrane contain electron-dense, slightly curved patches representing regions of the membrane at which envelopment takes place. Note the spikes projecting from the surface of the membrane of the capsid being enveloped. **B:** An enveloped capsid and numerous unenveloped capsids found late in infection in the cytoplasm of infected cells. Some of the capsids appear to be in the process of being either enveloped or de-enveloped. **C:** Micrograph showing an enveloped capsid in the space between the inner and outer lamellae of the nuclear membrane connecting with the cisternae of the endoplasmic reticulum. **D:** An unenveloped capsid in the nucleus and an enveloped particle bulging in the cisternae of the endoplasmic reticulum. **E:** Cytoplasmic enveloped particles enclosed in vesicles or cisternae of endoplasmic reticulum. **F:** Modified nuclear membranes folded upon themselves frequently seen in cells late in infection. The structures formed by such membranes have been designated as "reduplicated membranes." [Electron micrographs have been assembled from refs. 410 and 417 and from J. Schwartz and B. Roizman (*unpublished micrographs*).]

indicates the following: (i) An HSV-1 *ts* mutant expressing an altered gB attaches to, but does not penetrate into, cells (282); however, infection does ensue, and progeny virus is made after chemically induced fusion of the envelope of the adsorbed virus to the plasma membrane (441,442). Consistent with these results, gB^- virus attaches but does not penetrate (41). (ii) HSV-1 gD^- virus also attaches but does not penetrate (206). (iii) Cells expressing HSV-1 gD allow attachment and endocytosis of both HSV-1 and HSV-2; however, fusion of viral and cellular membranes, and

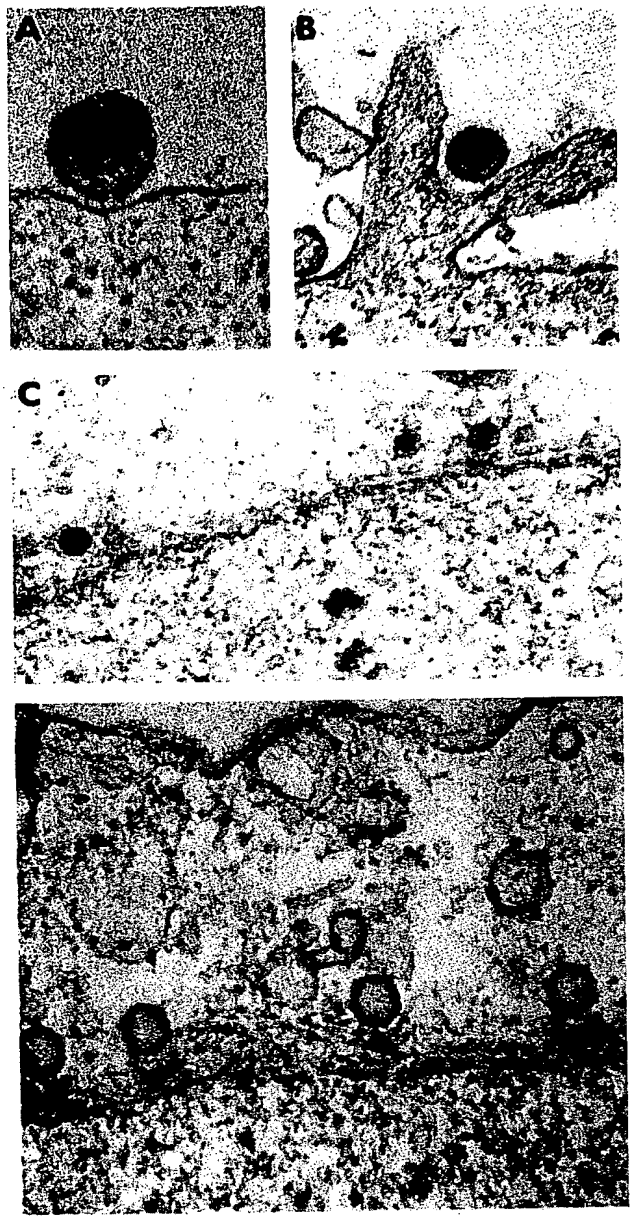

FIG. 6. Attachment and penetration of HSV-1 to cells in culture. **A** and **B:** Virions attached to plasma membrane. **C:** Capsids with DNA at nuclear pores in cells infected with HSV-1(HFEM)*ts*B7 maintained at the nonpermissive temperature (20). **D:** Empty capsids accumulating in cells late in infection with mutant HSV-1(50B) late in infection (505). In cells infected with this mutant, virtually every pore contains a juxtaposed empty capsid.

penetration, do not ensue (43). The interpretation of these results is as follows: (a) Both gB and gC recognize, as well as attach to, cell receptors; (b) gB and gD play an indispensable role in the fusion of the envelope with the plasma membrane; and (c) gD sequesters the cell membrane proteins required for fusion of the viral and cellular membranes. Virions attaching to the plasma membrane which cannot fuse are internalized and degraded in endocytic vesicles.

The transition from attached to penetrated virus, as measured by susceptibility to neutralization (characteristic of virus still attached to the cell surface), is very rapid (188).

Release of Viral DNA

Upon entry into the cell, the capsids are transported to the nuclear pores (Fig. 6) (20,505). Release of viral DNA into the nucleoplasm requires a viral function; thus, capsids of the *ts* mutant HSV-1(HFEM)*ts*B7 accumulate at nuclear pores and release viral DNA only after a shift down from nonpermissive to permissive temperature (20). Empty capsids are readily found at nuclear pores early in infection with wild-type viruses. The cellular cytoskeleton probably mediates the transport of herpesvirus capsids to the nuclear pores (80,244). Parental viral DNA accumulates in the nucleus.

Virion Components Required for Replication in Permissive Cells

Transfection of "permissive" cells with intact, deproteinated viral DNA yields infectious viral progeny (160,256,461). However, the specific activity of viral DNA is many orders of magnitude lower than that of virions, and the duration of the reproductive cycle is longer. Moreover, there is no certainty that the sequence of events in transfected cells resembles the viral reproductive cycle occurring in cells infected with competent virions.

The components of the virion other than its DNA appear to have several functions. In addition to protecting and facilitating the entry of the DNA into cells, the virion components appear to be involved in the early shut-off of host macromolecular synthesis (125,252,253,333–335,395,414,443,491,494–496). That virion components also participate in viral replication is deduced from the conclusion that a virion tegument protein (designated in the Spear and Roizman (478) nomenclature as VP16) acts in *trans* to induce α genes, the first set of genes to be expressed (21,46,354,367). Since the induction of α genes is a nuclear event, it is evident that at least some virion components make their way into the nucleus. Preston and Notarianni (376) reported ADP ribosylation of the capsid protein VP23 (148,150,478) in nuclei of freshly infected cells, suggesting that it is also translocated into the nucleus. It has previously been reported that phosphate cycles on and off VP23, suggesting either that VP23 is a component of the protein kinase associated with HSV vir-

ions or that the kinase phosphorylates and dephosphorylates VP23 and substrate proteins (265).

Viral Genes: Pattern of Expression and Characterization of Their Products

Timing and Requirements for Gene Product Synthesis

The transcription of viral DNA takes place in the nucleus. As would be expected, all viral proteins are synthesized in the cytoplasm. The number of abundant (i.e. readily detectable) polypeptides specified by HSV does not exceed 50 (76,183,324). Assuming that only the open-reading frames identified by McGeoch et al. (294,299) are expressed, the HSV genome would encode 70 polypeptides. However, as discussed later in this text, the definition of open-reading frames in the viral genome is somewhat arbitrary, and a higher number is not unlikely.

In cells productively infected with HSV, the regulation of viral gene expression schematically represented in Fig. 4 has three features: (i) HSV proteins form several groups whose synthesis is coordinately regulated in that they have similar requirements for, as well as similar kinetics of, synthesis; (ii) the absolute rate of synthesis and ultimate abundance of each protein may vary; and (iii) the protein groups are sequentially ordered in a cascade fashion (124,183–185, 241,357).

α genes are the first to be expressed. There are five α proteins, namely, infected cell polypeptides (ICPs) 0, 4, 22, 27, and 47. α genes were initially defined as those that are expressed in the absence of viral protein synthesis. The α genes may be defined more precisely by the presence of the sequence 5′ NC GyATGnTAATGArATTCyTTGnGGG 3′ in one to several copies within 400 base pairs upstream of the cap site (280). We should also note that in addition to the five α genes, other domains of the viral genome are transcribed under "α" conditions. The two reported to date are: (i) the latency-associated transcript 1 (LAT1), which is antisense to, as well as partially overlapping, the 3′ domain of the α0 gene (486); and (ii) a transcript designated as ORI_sRNA_1, located in the inverted repeats of the small component with a start site within the 5′ transcribed noncoding domains of the α22/α47 genes, which extends antisense to those genes and terminates approximately at the cap site of the α22/α47 mRNAs (190,191).

The synthesis of α polypeptides reaches peak rates at approximately 2–4 hr post-infection, but α proteins continue to accumulate until late in infection at nonuniform rates (184). To date, all α proteins, with the possible exception of α47, have been shown to have regulatory functions. As discussed in detail below, functional α proteins are required for the synthesis of subsequent polypeptide groups.

β genes are not expressed in the absence of competent α proteins; moreover, their expression is enhanced, rather than reduced, in the presence of inhibitory concentrations of drugs that block viral DNA synthesis or in cells infected with tight DNA^- *ts* mutants in β genes. The β_1 and β_2 groups of polypeptides reach peak rates of synthesis at about 5–7 hr post-infection (184). β_1 genes, exemplified by polypeptides $\beta_1 6$ [the large component of the viral ribonucleotide reductase (192)] and $\beta_1 8$ [the major DNA-binding protein (68)], appear very early after infection and have previously been mistaken for α proteins (61). They are differentiated from the latter by their requirement for functional α4 protein for their synthesis (184,185). β_2 polypeptides include the viral thymidine kinase (TK) and DNA polymerase. The appearance of β gene products signals the onset of viral DNA synthesis, and most viral genes involved in viral nucleic acid metabolism appear to be in the β group.

γ genes have been lumped for convenience into two groups, γ_1 and γ_2, although in reality they form a continuum differing in their timing and dependence on viral DNA synthesis for expression (68,72,176,177, 217,466,515). The prototype γ_1 gene (e.g., the genes specifying glycoproteins B and D) is expressed relatively early in infection and is only minimally affected by inhibitors of DNA synthesis. The relatively abundant major capsid protein, $\gamma_1 5$, is made both early and late in infection. In contrast, prototypic γ_2 genes are expressed late in infection and are not expressed in the presence of effective concentrations of inhibitors of viral DNA synthesis.

γ_1 genes have also been designated as βγ genes (72,176,177). The differentiation of β genes into β_1 and β_2 and the variability in the requirements for the expression of γ genes are the major reasons for the designation of HSV genes as α, β, and γ rather than immediate-early, early, and late (184).

The Functional Organization of Herpes Simplex Virus Genomes

The sources of the data for the functional organization of the HSV-1 genome shown in Fig. 7 are useful to present for both historical and heuristic reasons. Globally, the key sources were the transcriptional maps painstakingly collected and defined by Wagner and associates (5–7,71,72,74,101,102,139,140,162,176, 177,515). These maps served as the basis for the interpretation of the nucleotide sequence data generated by McGeoch and associates (294–300); however, in some instances, transcriptional analyses (and even translational analyses) were ignored in favor of nu-

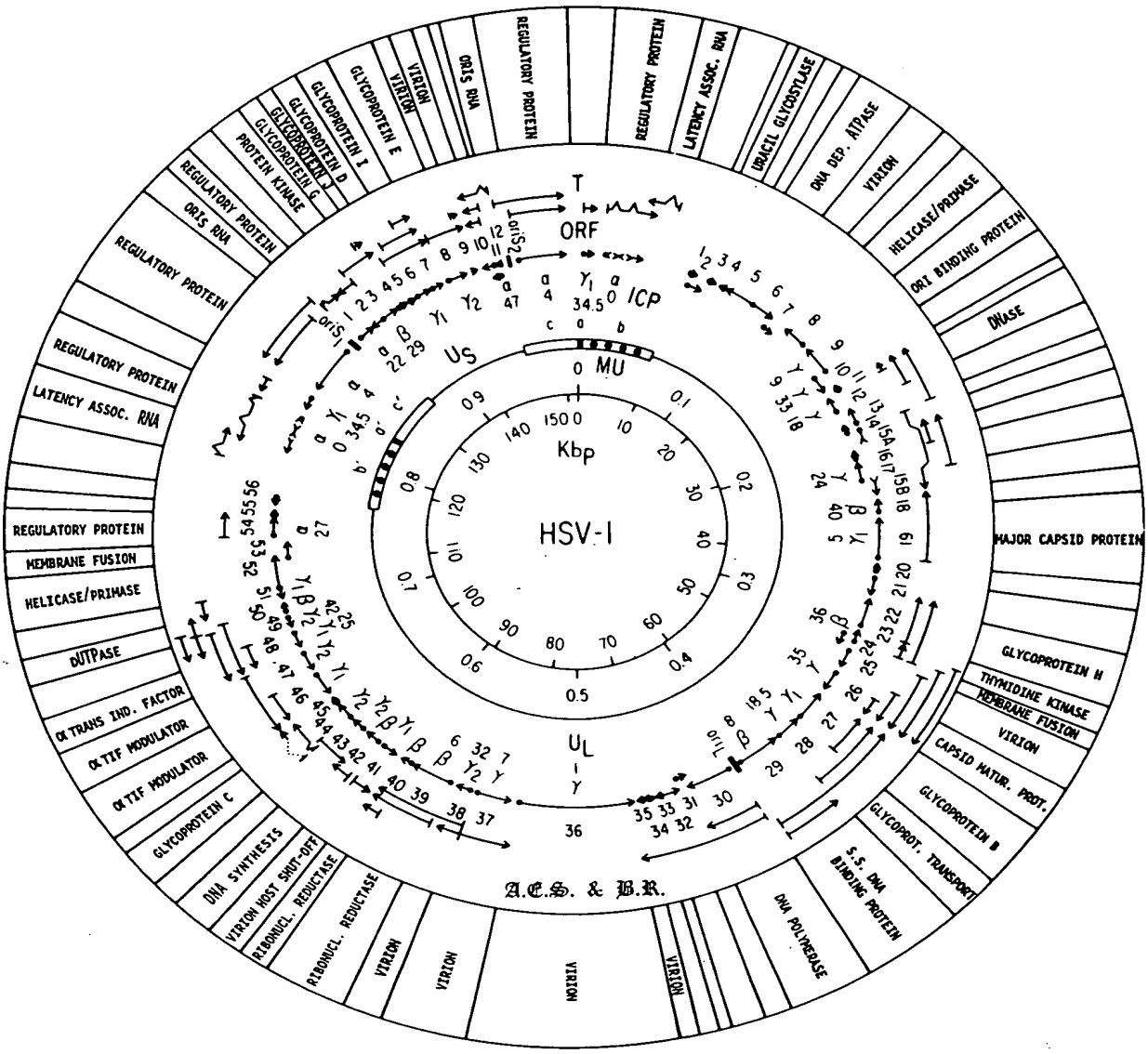

FIG. 7. Functional organization of the HSV-1 genome. The circles are described from inside out. *Circle 1*: Map units and kilobase pairs. *Circle 2*: Sequence arrangement of HSV genome shown as a circularized version of the P arrangement. Cleavage of the circle at O map units would yield a linear molecule in the P arrangement. The letters *a*, *b*, *c*, U_L, and U_s identify different domains of the genome. *Circle 3*: Representation of the open-reading frames. The letters and numbers indicate the regulatory class (α, β, γ_1, or γ_2) to which the gene belongs, and they also indicate the ICP designation of the product. The numbers outside the circle indicate the open-reading frames according to McGeoch et al. (294,298). *Circle 4*: This represents the direction and approximate size of the transcripts as described by numerous laboratories. *Circle 5*: This lists the known functions of the proteins specified by the open-reading frames. Note that virion structural proteins are listed either as "glycoprotein" or "virion." The genes dispensable for growth in cells in culture are listed in the text. The data for circles 3 and 4 are derived from refs. 6, 39, 57, 71, 72, 74, 82, 86, 89, 101, 113, 139, 146, 162, 178, 296–300, 304, 305, 310, 311, 330, 353, 354, 358, 393, 398, 400, 401, 459, 524, and 532. The references for gene functions (circle 5) are listed in the text.

cleotide sequences denoting putative transcriptional initiation sites or terminations. Identification of the proteins specified by the individual open-reading frames is based on several sources. The framework and much of the initial mapping of the HSV genome are based on analyses of proteins and DNA sequence arrangements of HSV-1 × HSV-2 recombinants (286,323,324,434) supplemented by (a) rescue of mutants by transfection of cells with intact mutant viral DNA and DNA fragments generated by restriction endonuclease digestion of wild-type genomes (see, e.g. refs. 232, 233, 326, and 348), (b) transfer of a dominant or assayable marker from one genome to another with restriction endonuclease fragments (see e.g., refs. 232, 240, 367, 368, and 434), and (c) expression of the gene product from purified mRNA or from a DNA fragment in a suitable system (see, e.g., 68, 172, 262, 277, and 367). The products of a large number of putative open-reading frames have not been identified. The sequence-dependent, in contrast to transcription- or function-dependent, identification of open-reading frames is conservative and does not take into account proven exceptions (e.g., the arbitrary rules would have excluded α0 as an open-reading frame if its product had not been known). Nevertheless, the overall organization of the genome is becoming apparent and can be summarized as follows:

1. α genes map near the termini of the L and S components (5,218,277,324,330,378,522,523); α0 and α4 map within the inverted repeats of the L and S components, respectively, and are therefore each present in two copies per wild-type genome. However, a single copy of each is sufficient inasmuch as HSV-1 (I358), a mutant lacking most of the internal inverted repeat sequences, is viable (363). In the circular arrangement of viral DNA, the α genes form two clusters. The first consists of α genes, 0, 4, and 22, whereas the second consists of α genes 47, 4, and 0. A key feature of these two clusters is that each contains an origin of DNA synthesis (Ori_s) sandwiched between α4 and α22 or between α4 and α47. Notwithstanding the clustering, each α gene has its own promoter-regulatory region and transcription initiation and termination sites (277–279).

2. β and γ genes are scattered in both the L and S components. At present, only two functional gene clusters are strikingly apparent, but their significance is uncertain: The β genes specifying the DNA polymerase and the DNA-binding protein flank the L component origin of DNA synthesis (Ori_L), and the γ genes specifying membrane glycoproteins D, E, G, I, and J map next to each other within the unique sequences of the S component (3,130,261,397,419,434,474,475, 524; N. Frenkel, *personal communication*). Although there are several instances of apparent sharing of 5′ or 3′ gene domains (102,398,522), there is altogether little gene overlap and few instances of gene splicing (515) relative to the frequency with which these events have been observed to occur in adenovirus and papovavirus genomes.

In this chapter, each protein shall be designated by one of three criteria: (i) by function, if it is precisely defined (e.g., thymidine kinase, DNA polymerase, etc.), (ii) by the first published designation of the protein, or (iii) by its open-reading frame.

Synthesis and Processing of Viral Proteins

Viral proteins appear to be made on both free and bound polyribosomes. Most of the proteins examined to date appear to be processed extensively after synthesis (2,33,45,112,148,186,357,473–476). Processing includes cleavage, phosphorylation, sulfation, glycosylation, and poly(ADP) ribosylation. In some instances the modifications in protein structure accompany the translocation of proteins across membranes (287). Current information concerning processing of proteins and the relationship of processing to function is detailed in the section on general properties and functions of viral proteins and in the section on viral glycoproteins.

With the exception of some glycoproteins, the extent to which processing is a requirement of virus growth rather than the consequence of a confrontation between cellular or viral enzymes and molecules resembling natural substrates remains uncertain.

It is noteworthy that HSV specifies a protein kinase whose gene maps in the S component (131,297, 388,389). The gene is dispensable for growth in cell culture, and its function is not known. It has been suggested that HSV may also encode a glycosyl transferase, but to date this has not been demonstrated.

Application of Genetic Techniques to the Identification of Gene Product Function: Genes Essential and Nonessential for Growth in Cell Cultures

Key to the identification of viral functions and mapping of viral genes encoding these functions are temperature-sensitive (*ts*) mutants. Some 30 complementation groups have been identified to date (see ref. 529)—an extraordinary accomplishment in itself, given the difficulties inherent in the selection and testing, as well as in the placement of the mutants into complementation groups. The *ts* mutants have been enormously helpful in mapping genes. Nevertheless, this approach to identification and mapping of viral functions suffers from several problems: (i) The pheno-

types of viruses containing extensive mutations in some nonessential genes cannot be readily differentiated from that of wild-type virus; (ii) conditional lethal (e.g., *ts*) mutants produced by general mutagenesis of the viral genome may contain a large number of silent nonlethal mutations in both essential and nonessential genes; (iii) the phenotypes of mutations introduced into domains shared by more than one gene cannot be readily attributed to the malfunction of a specific gene product; and (iv) while the usefulness of *ts* mutants is, in part, dependent on their efficiency of plating at permissive and nonpermissive temperatures, tight mutants with high permissive/nonpermissive ratios may well contain more than one point mutation. Although the presence of multiple mutations in a single gene should not affect the mapping or identification of the gene function, it does present a problem in mapping the functional domains of the gene.

An alternative to the random or fragment specific substitution of bases in DNA is site-specific deletion of the viral genome. A protocol for site-specific insertion/deletion of viral genes was first reported by Post and Roizman (368). It was based on selection of recombinants generated by double recombination through homologous flanking sequences between an intact viral DNA molecule and a DNA fragment containing an insertion or deletion and a selectable marker. The selectable marker used in these studies was the viral thymidine kinase (*tk*) gene because (i) it can be deleted from the HSV genome without affecting growth of the virus in cell culture, (ii) a plasmid-borne *tk* gene can be altered so that it cannot recombine by double crossover to repair the deletion in the genomic *tk* gene, (iii) viruses carrying a functional *tk* gene can be selected against by plating viral progeny in the presence of nucleoside analogues phosphorylated by the viral TK (e.g., Ara T), and (iv) viruses expressing the *tk* gene can be selected for by plating the virus in TK^- cells in medium containing methotrexate or aminopterin, which block the conversion of TMP from dUMP by thymidylate synthetase and preclude the *de novo* pathway of TMP synthesis. This procedure permits the selection of viable mutants with deletions or insertions in genes that appear to be nonessential for growth in cells in culture. Other investigators adapted the double-crossover protocol for selection of mutants with deletions in essential genes (41,94,267). In this protocol the gene to be deleted was transfected into, and expressed in, Vero cells; the vector cell line (i.e., the cells expressing the gene) was then transfected with intact viral DNA and the mutated DNA fragment. The progeny of transfection were screened for deletion mutants that multiplied only in the vector cell line.

A still different protocol for insertional mutagenesis is based on the use of transposons (e.g., miniMu phage, *Tn5*) (202,418,526). Its principles were described first by Jenkins et al. (202), taking advantage of the random insertion of miniMu into target plasmid DNAs. A miniMu phage containing a modified HSV-1 *tk* gene was constructed. Transposition of this miniMu into an HSV fragment is random and is limited to one insert per plasmid copy. Transfection of intact TK^- viral DNA with an HSV DNA fragment containing random insertions of the modified miniMu would result in recombinants in which the miniMu randomly inserted into the viral DNA fragment would become recombined at the identical position in the viral genome. However, only the genomes containing the miniMu at a nonessential site multiplied in cells in culture.

Among the genome domains not essential for growth in cells in culture are the following: all of the genes mapping in U_s except for that specifying glycoprotein D (273–275,526); the internal inverted repeats (203,274,363); one origin of DNA synthesis in the S component (274); the origin of DNA synthesis in the L component (530); α0 (437,489); dUTPase (128); uracil-DNA glycosylase (327); glycoprotein C (170, 175); the major component of the ribonucleotide reductase (ICP6) (42,154); the minor component of the ribonucleotide reductase (42); the thymidine kinase (223,368); a gene reported to cause fusion of cells (UL24) (196); a gene 3′ to αTIF reported to modulate its activity (UL44) (J. McKnight and B. Roizman, *unpublished data*); and the genes mapping between the 3′ end of α27 and the internal inverted repeats (313). Among the essential genes deleted from the viral genome are those specifying glycoprotein B (41), glycoprotein D (267), α4 (94, 471), α27 (291), and ICP8 (343). As expected, gB^-, gD^-, $ICP8^-$, $\alpha27^-$, and $\alpha4^-$ viruses are not capable of yielding infectious progeny in cells that do not express gB, gD, ICP8, ICP27, or ICP4.

In a special category are deletion mutants whose ability to multiply is cell-species-dependent. One example of such mutants is the $\alpha22^-$ virus, which grows well in Vero and HEp-2 cell lines but not in human fibroblast strains or in rodent cell lines (450). In the nonpermissive cells, the virus fails to express γ_2 genes efficiently.

It could be predicted that viral genes that specify products whose functions are identical and interchangeable with those of cellular genes would be dispensable, at least in cells that express these functions. In this category are the *tk* gene, the genes specifying ribonucleotide reductase, and possibly the gene specifying the protein kinase. It is conceivable that the functions of other viral genes (e.g., those of α22) are complemented by some cells but not by others, and it is also conceivable that some of the dispensable genes may also be complemented by cellular counterparts. However, the dispensable genes specifying the surface glycoproteins C, E, G, I, and J must be in a different

category. It is difficult to ascribe to these proteins functions other than those associated with entry into cells or with egress from cells, functions that are also ascribed to glycoproteins B, D, and H. While we cannot exclude the possibility that cells express proteins with similar functions which complement the deletion mutants, a more likely scenario is that HSV itself carries a set of genes which enables the virus to multiply in a wide variety of human cells. Among these cells may be not only those that do not express *tk*, ribonucleotide reductase, and so on, but also those which require alternate pathways for infection, for regulation of gene expression, and so on. These "nonhomologous" functional alternates remain to be established, but it is relevant to reiterate that both glycoproteins B and C endow the virus with the capability of attaching to heparan sulfate proteoglycans (547).

Synthesis of Viral DNA

Temporal Pattern of Synthesis

A characteristic of herpesviruses not shared by other nuclear DNA viruses is that they specify a large number of enzymes involved in DNA synthesis. Although the sequence of events in viral DNA replication is roughly known, details are lacking. In HSV-infected cells, viral DNA synthesis is detected at about 3 hr post-infection and continues for at least another 9–12 hr (413,414,421). The DNA is made in the nucleus, but the kinetics of DNA synthesis have not been addressed properly. Earlier studies relied on incorporation of labeled thymidine into viral DNA—a procedure that yielded biased results inasmuch as the deoxynucleotide triphosphate pool increases and becomes saturated early in infection. Hence, the rate of viral DNA synthesis as determined by the use of labeled deoxynucleosides appears to be highest relatively early in infection.

Structure of Replicating DNA

At least in HSV-1-infected cells, only a small portion of total input (parental) viral DNA is replicated (194). The DNA labeled during a pulse lacks free ends; that is, it consists of circles or head-to-tail concatemers (194,195). Labeled precursors become incorporated into molecules banding at a higher density which sediment at a faster rate than intact double-strand DNA. In alkaline sucrose density gradients, the bulk of the labeled DNA bands at a position expected for small single-strand fragments. Early after the onset of viral DNA synthesis, parental DNA, circles, and linear branched forms can be found in the DNA banding at the density of viral DNA. These are replaced late in the reproductive cycle by large, rapidly sedimenting bodies of tangled DNA. Available evidence suggests that, at least late in infection, herpesvirus DNAs replicate by a rolling circle mechanism (24,195). Attempts to find "theta" forms of replicating DNA early in infection have not been successful.

Origins of DNA Replication

The origins (*ori*) of DNA replication in the HSV genome (271) were initially deduced from the structures of defective genomes (134,446,471a) and have more recently been operationally defined as those sequences which must be present in a fragment of HSV DNA in order for it to be amplified in permissive cells transfected with the fragment and either transfected or infected with helper virus (319,512). By this definition, HSV-1 and presumably HSV-2 each contain three origins of DNA replication. Two of the origins map in the *c* reiterated sequence of the S component, between the promoters of α4 and α22 (ori_s1) or α4 and α47 (ori_s2) (17,88,319,487,488,512), whereas a third origin (ori_L) maps in the middle of the L component sandwiched between the promoters of the β genes specifying the major DNA-binding protein (ICP8) and the DNA polymerase (271,472,530).

The L-component origin consists of an A+T-rich, 144-base-pair sequence forming a perfect palindrome (234,272,393,530). Because of its extensive dyad symmetry, it tends to be unstable in DNA fragments cloned in *Escherichia coli* (530). The S component origin is shorter and contains a much shorter A+T-rich palindrome which is related to, but lacks, the complete dyad symmetry of ori_L. It has been suggested that the structure of ori_L enables bidirectional synthesis, whereas DNA synthesis initiated in ori_s would be asymmetric. The existence or necessity for bidirectional synthesis of DNA remains to be established. Ori_L and at least one ori_s can be deleted without affecting the ability of the virus to multiply.

Very little is known about the function of the origins. The conclusion that these sequences are indeed origins is supported by the studies of origin-dependent amplification of DNA by fragments encoding genes shown to be essential for viral DNA synthesis. However, major questions remain unanswered, particularly the function of the origins once viral DNA synthesis is initiated and whether the two kinds of origins are equivalent or subordinate to each other, especially because the reported positions of loops in replicating HSV DNA appear, in some instances, to be different from those of the known origins (465). It is noteworthy that both ori_L and ori_s are situated between transcription initiation sites. The locations of the origins suggest that initiation of DNA synthesis might be activated, or

at least enhanced, by the changes in the local environment of the DNA due to transcription initiation events.

Functional Requirements for DNA Synthesis

HSV specifies a large array of proteins involved in nucleic acid metabolism and DNA synthesis. These proteins fall into two categories: (i) proteins that are essential for viral origin-dependent amplification of DNA and (ii) enzymes involved in nucleic acid metabolism (e.g., thymidine kinase, ribonucleotide reductase, dUTPase, uracil-DNA glycosylase, alkaline exonuclease) which, for the most part, appear not to be essential for viral growth in cells in culture. A virus-specific topoisomerase has also been reported (329) but has not been shown to be virally encoded (16).

Much of the initial evidence for viral proteins essential for DNA synthesis emerged from studies of the defects in DNA$^-$ *ts* mutants. More recently, the genes whose products are essential for DNA synthesis were identified by transfecting cells with a plasmid containing an origin of DNA synthesis and various fragments of the HSV genome. These studies (53) identified seven genes mapping in the L component (open-reading frames UL5, UL8, UL9, UL29, UL30, UL42, and UL52) required for viral-origin-dependent DNA synthesis. The seven genes specify the following: a DNA polymerase (UL30) with an apparent molecular weight of 140,000 (55,62,167,182,220,221,369); a single-strand specific-DNA-binding protein designated as ICP8 (UL29) with an apparent molecular weight of 124,000 (53,59,68,204,237,283,288,369,390,435,546); a protein binding to the origin of viral DNA synthesis (UL9) (106,107,236,340) with a translated molecular weight of 94,000 (107); a protein that binds to double-strand DNA (UL42) with a molecular weight of 62,000 (285,294,347,546); and three additional proteins (UL5, predicted molecular weight of 99,000; UL8, predicted molecular weight of 80,000; and UL52, predicted molecular weight of 114,000). These three proteins form a complex in which each protein is present in equimolar rations and which functions as a primase and a helicase (78). An HSV-specific primase activity was previously reported by Holmes et al. (179). The protein specified by UL5 has independently been shown to be a DNA-dependent ATPase (E. Mocarski, *personal communication*).

The DNA polymerase, in particular, has been the object of numerous studies because of its unusual sensitivity to a variety of compounds (e.g., phosphonoacetate and phophonoformate). Temperature-sensitive mutants expressing altered HSV DNA polymerases have been described (55,62,386,387), and some mutants have been found to be resistant to a variety of drugs such as phosphonoacetate (54,204,386) and nucleoside analogues [e.g., acycloguanosine (77)] inhibitory to wild-type viruses.

ICP8 ($\beta_1$8) has also been extensively investigated, particularly by Knipe and colleagues (151–153,237,259,260,390–392), and by Ruyechan and colleagues (431–433,435). The protein has an apparent molecular weight of approximately 120,000. It has an affinity for single-strand DNA (385), and its binding is cooperative (431). Temperature-sensitive mutants in this gene fail to synthesize viral DNA at the nonpermissive temperature (68,151), as do deletion mutants in cells that do not provide ICP8 in *trans* (343).

The seven proteins described above appear to be all that is necessary for ori$_s$-dependent amplification of DNA transfected into cells. Other proteins undoubtedly play a role in processing, cleavage, and packaging of the genomic viral DNA, as well as in the production of precursors of DNA synthesis; for example, as described below, the alkaline DNase is not among these seven genes but is essential for viral DNA replication (325).

Alkaline DNase

HSV-1 induction of an alkaline DNase activity was first reported in 1963 (221). The gene has been mapped by transient expression in oocytes (373) and by the use of HSV-1 × HSV-2 recombinants (14) to between 0.145 and 0.185 map units, corresponding to open-reading frame UL12 (294). The protein is encoded by a 2.3-Kb mRNA (101) and has a predicted translated molecular weight of 67,503 (294) and an apparent molecular weight of 80,000–85,000 (15). A *ts* mutant (132) was used to demonstrate that the DNase activity is essential not only for viral growth but also for DNA replication (325). In contrast to the requirement for this activity for DNA synthesis in infected cells, the enzyme is not required in transient expression assays involving individual viral genes and a viral origin of DNA synthesis contained in a plasmid (53).

Several other proteins involved in nucleic acid metabolism have been described but appear not to be essential for virus growth in cells in culture.

Thymidine Kinase (TK)

TK is by far the best known of the viral proteins. A unique characteristic of TK is that its substrate range is far greater than that of its host counterpart. Although it has been designated as a deoxypyrimidine kinase (198), it actually phosphorylates purine pentosides and a wide diversity of nucleoside analogues that are not phosphorylated efficiently by cellular kinases (204, 224,231). This characteristic of TK is the basis for the

effectiveness of various nucleoside analogues in the treatment of experimental and natural herpesvirus infections. The observation that TK is essential for normal virus multiplication in experimental infections (127,500) but not in cell culture (223) is the basis of much of the probing of the HSV genome structure done in recent years (363,367,368). Mutants in the *tk* gene fall into several groups. Some fail to produce functional TK altogether, whereas others make either (a) reduced amounts of enzyme or (b) an enzyme with an altered substrate specificity which is resistant to the analogue used in the selection process (83,127, 370,492).

Ribonucleotide Reductase

The HSV ribonucleotide reductase consists of two proteins: The large subunit, ICP6 (184,185,192,380), has an apparent molecular weight of 140,000 and a predicted translated molecular weight of 124,043 (294); the small subunit has an apparent molecular weight of 38,000 (11,380) and a predicted translated molecular weight of 38,017 (294). The two genes are encoded by 3′-coterminal mRNAs of 5.0 Kb for the large subunit and 1.2 Kb for the small subunit (6,493). The two proteins are tightly associated in a $\alpha_2\beta_2$ complex (11,12,193), and both subunits are required for activity (11,129,187).

Ribonucleotide reductase functions to reduce ribonucleotides to deoxyribonucleotides, creating a pool of substrates for DNA synthesis. The viral enzyme is not essential for growth in actively dividing cells maintained at 37°C (154). However, it is required for efficient viral growth and DNA replication in nondividing cells or in cells maintained at 39.5°C (155,380), indicating that at 37°C, actively dividing cells can complement the viral function.

Uracil-DNA Glycosylase

HSV encodes a uracil glycosylase, which presumably functions in DNA repair and proofreading. Uracil glycosylase acts to correct the insertion of dUTP and to correct the deamination of cytosine residues in DNA; the extremely high G+C content of HSV DNA makes this an important element of error correction in HSV DNA replication. The HSV-induced uracil glycosylase has been identified by Caradonna and Cheng (47), and its coding domain was initially mapped to between 0.065 and 0.08 map units (48), a location correlating to the UL2 open-reading frame (294). Subsequent *in vitro* translation experiments definitively identified UL2 as the uracil glycosylase gene (545). The protein has an apparent molecular weight of 39,000 (48) and a predicted translated molecular weight of 36,326 (294). The gene has been deleted and is nonessential for growth of the virus in culture (327).

dUTPase (Deoxyuridine Triphosphate Nucleotidohydrolase)

dUTPases act to hydrolyze dUTP to dUMP, providing both (a) a mechanism to prevent incorporation of dUTP into DNA and (b) a pool of dUMP for conversion to dTMP by thymidylate synthetase. An HSV-encoded dUTPase has been identified (47,543); contrary to early reports (543), the purified enzyme is specific for the hydrolysis of dUTP (541). The viral gene has been mapped to between 0.69 and 0.70 map units by transient expression (379), corresponding to the UL50 open-reading frame (294). dUTPase activity appears to be lacking in HSV-1(17)*ts*K, a mutant in ICP4 used for analyses of gene regulation (84). The dUTPase gene has subsequently been shown to be nonessential for growth of the virus in tissue culture (128).

Assembly of Capsids

Capsids are assembled in the nucleus (Fig. 3). The steps in the assembly are not defined. Viral DNA is packaged into preformed capsids.

Cleavage and Packaging of HSV DNA

Newly synthesized viral DNA is "processed" and packaged into preformed empty capsids. "Processing" involves (a) amplification of *a* sequences and (b) cleavage of viral DNA lacking free ends (i.e., in circular or head-to-tail concatemeric form). The isomerization of the DNA is associated with the process of DNA replication, cleavage, and packaging. There is considerable evidence that cleavage and packaging of DNA are linked processes (90,91,254,255). The isomerization of the DNA is less well understood. The available data have come from three sources: (i) analyses of the termini of standard viral genomes (87,321,323); (ii) analyses of termini of viral genomes containing insertions of additional *a* sequences (56,510); and (iii) studies on amplicons, plasmids containing an origin of viral DNA synthesis and one or more *a* sequences which are amplified and packaged with the aid of a helper virus (90,91,472,512,513).

The net result of the process of cleavage of standard genomes from concatemers is the generation of (a) a free S-component terminus consisting of one *a* sequence with a terminal DR1 sequence containing only a single base pair and and one 3′ nucleotide extension (320) and (b) a free L-component terminus consisting of one to several directly repeated *a* sequences and

ending in a DR1 containing 18 base pairs and one 3′ nucleotide extension. Upon circularization of the DNA following entry into cells, the two partial DR1 sequences together would form one complete DR1 shared by two *a* sequences. In the reverse process of linearization of viral DNA for packaging, cleavage of endless (circular or concatemeric) DNA occurs asymmetrically within a DR1 second distal from the *c* sequence and, in an ideal case, shared by two *a* sequences. Studies on junctions containing a single *a* sequence show that they are cleaved (91). The results of such studies have been interpreted to indicate either (a) that the sequence *xay* is cleaved to yield *xa* and *y*, and the *y* product is processively degraded along the DNA to the next *a* sequence, or (b) that the cleavage simultaneously yields both *xa* and *ay* by amplification of the *a* sequence during the cleavage process (90,91,510). Parenthetically, there is little doubt that DNA lacking a terminal *a* sequence could be degraded, but inasmuch as nearly 50% of the L–S component junctions are of the *bac* type (i.e., have a single *a* sequence), a hypothesis whose logical extension is that 50% of newly synthesized DNA is degraded during packaging does not make biological sense.

Deiss et al. (90) analyzed the cleavage and packaging of a series of amplicons. Those lacking the *Ub* sequence were amplified and packaged, but they acquired an intact *a* sequence from the helper virus. Those lacking the *Uc* sequence were not subject to cleavage and packaging. Domains were identified in *Ub* and *Uc* which were conserved in several herpesviruses and which were designated *pac1* and *pac2*, respectively. The model (90) that best fits the data, presented here in a slightly modified form (Fig. 8), consists of several steps: (i) A cleavage–packaging protein attaches to the *Uc* sequence. (ii) A putative structure on the surface of the capsid complexes with a *Uc*-bound protein sequence, loops the viral DNA, and scans from the bound *a* sequence (a_1) across the L component toward the end of the S component until it detects the first Uc-DRI-Ub domain of an *a* sequence in an identical orientation. (iii) In the juxtaposed *a* sequences, the DR1 sequence of one *a* is cleaved and the gap is repaired, resulting in the generation of an *a* sequence by the mechanism proposed by Szostak et al. (497) to explain recombinational events resulting in gene conversion. (iv) Cleavage then occurs in the DR1 shared by the two *a* sequences. In this model, the *a* sequences in the internal inverted repeats play no role in the packaging of the unit-length molecule, consistent with the observation that HSV-1 DNA from which the internal inverted repeats are deleted do package effectively.

The packaging component of the model of Deiss et al. (90) predicts that the length of the packaged DNA can be defined by the distance between two directly repeated *a* sequences. The studies by Frenkel et al.

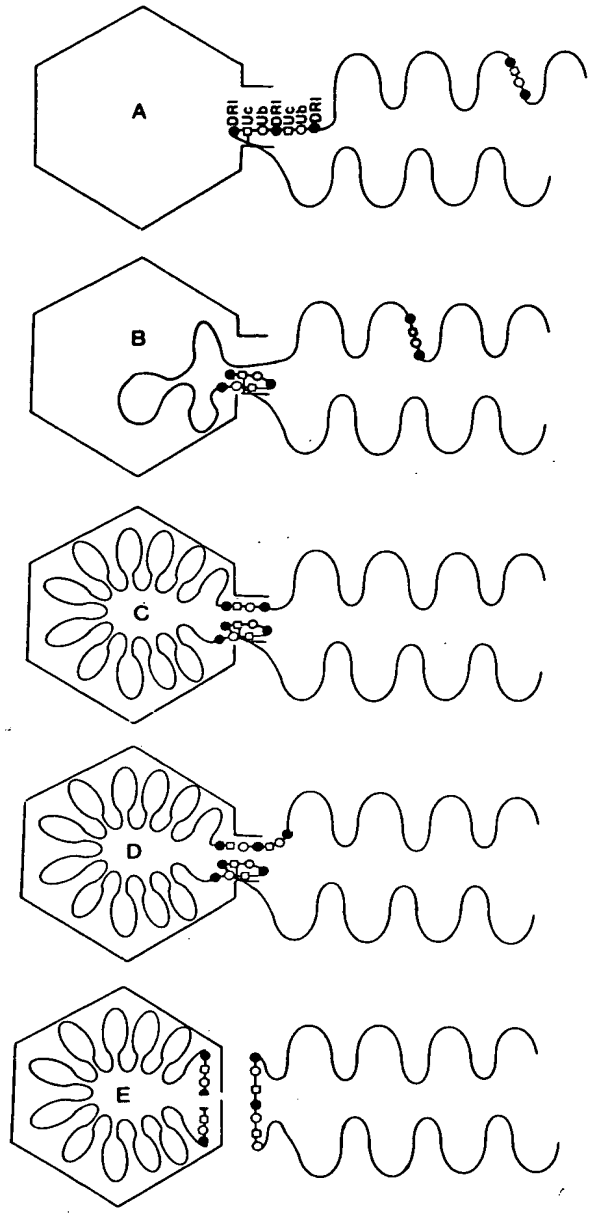

FIG. 8. Packaging of HSV-1 DNA. The current model developed by N. Frenkel and associates is described in Deiss et al. (90) and in the text. The model requires that proteins attach to components of the *a* sequence, probably Uc, and that empty capsids scan concatemeric DNA until contact is made in a specific orientation with the first protein–Uc sequence (**capsid A**); the DNA is then taken into the capsid (**capsid B**) until a "head full" is taken in or one *a* sequence whose nucleotide arrangement is in the same orientation (i.e., one genome equivalent in length away) is encountered (**capsid C**); the packaging signal requires nicking of both strands from signals on opposite sites of a DR1 sequence. In the absence of two adjacent *a* sequences (**capsid D**), the juxtaposition of the *a* sequences would result in duplication of the *a* sequence (**capsid E**) as described by Deiss et al. (90).

(134) indicate that the situation is likely to be more complex. Defective genomes consisting of 17+ direct reiterations of a unit consisting of an *ori* and an *a* sequence are readily detected in virions of HSV stocks derived by serial passages at relatively high multiplicities (134). These observations are consistent with the hypothesis that, besides the scanning mode, there is a "head full" recognition element that selects the juxtaposed *a* sequence once a threshold amount of DNA has been packaged. There is evidence, however, that shorter fragments of HSV DNA are packaged into capsids but that these capsids do not become enveloped (513). The hypothesis that may explain the apparent contradiction is as follows: Packaging aborts when the DNA reeled into the capsid is smaller than full length, but the capsid does not disgorge the packaged fragment.

The viral proteins responsible for the cleavage–packaging event have not been identified, but two sets of DNA-binding proteins of potential significance have been reported. Chou and Roizman (58) have identified two viral proteins which form a sequence-specific complex with the portions of the *Uc* sequence containing *pac2*. In addition, capsids contain a protein that binds viral DNA [VP19C or ICP32 (32)].

Inversions of the L and S Components

The isomerization of HSV DNA resulting from the inversion of the L and S components relative to each other is an intriguing, tantalizing feature of the HSV genomes shared with only a few other herpesviruses.

In its circular form, the HSV genome forms two isomers, each containing two L–S component junctions. Cleavage of one circular isomeric form at the two junctions would yield the P and I_{SL} arrangements, whereas the corresponding cleavages of the other circular isomer would yield the I_S and I_L isomers. Generation of the I_S and I_L arrangements from the first circular isomeric form would require inversion of either the S or the L component through the inverted repeat sequences.

Fundamentally, there are several issues. First, inversion of covalently linked components is not a property of all herpesvirus genomes. Second, the physiologic function of the inversions is not clear inasmuch as genomes frozen in one orientation as a consequence of deletion of internal inverted repeats are viable (203,362). However, all wild-type isolates examined to date do contain the inverted repeat sequences, and viruses lacking internal inverted repeats have a reduced capacity for growth in animal tissues. Third, insertion of the junction between the L and S components, and especially of the 500-base-pair *a* sequence, results in additional inversions of DNA segments contained between inverted repeats of *a* sequences (56,317, 321,323). Deletion analyses have shown that inversions are associated with the sequences DR2 and DR4; deletion of these sequences results in a gross reduction in the inversion frequency (56). Lastly, inversion of viral DNA segments flanked by other domains of the genome, or inversion between repeated foreign DNA sequences, was observed in some instances but not in others. In the case of fragments duplicated in different components of the HSV DNA, the segment of the genome flanked by the inverted repeats does not invert (317,368). DNA fragments flanked by inverted repeats contained in the same component do invert. In some instances, the inversions were accompanied by a high-frequency gene conversion (365). Recently, Weber et al. (525) reported that inversions of DNA segments flanked by inverted Tn5 transposon elements resulted from recombination events through homologous sequences and was not the consequence of a recombinational event mediated at a specific *cis*-acting site by *trans*-acting viral proteins. Thus, inversions of amplified DNA sequences flanked by inverted Tn5 sequences at least 600 base pairs or longer were noted in cells transfected with the genes specifying the seven proteins required for viral DNA synthesis. As in the case of amplicons containing two *a* sequences in an inverted orientation (319), inversions were not observed in the absence of DNA synthesis. Experiments with HSV L–S junctions were also done, but only with direct repeats of the *bac* sequence. In this instance, the intervening segment was deleted.

The central issue is not that DNA sequences flanked by inverted repeats tend to invert as a consequence of homologous recombination, but rather the frequency of such inversions. The DNA extracted from a plaque, generated by a single virus particle presumed to be in one arrangement of DNA, contains all four isomers of HSV DNA in equimolar concentrations. In the case of DNA segments flanked by inverted repeats of nonjunction fragments, the fraction of the genomes showing inversions even after many serial passages is seldom more than a small fraction of the total. The accumulated and predicted evidence that DNA segments flanked by inverted repeats in HSV genomes can invert does not resolve the mystery of the high-frequency inversion of the L and S components relative to each other during viral replication.

Envelopment

Entry of Viral Proteins into Cellular Membranes

The hallmark of infected cells late in infection is the appearance of reduplicated membranes and thick, concave or convex patches, particularly in nuclear mem-

branes (Fig. 5) (69,111,263,293,321,332,447,464). Nuclear envelopment takes place at these patches. Because the enveloped virions do not contain detectable amounts of host membrane proteins, it is likely that the patches represent aggregations of viral membrane proteins, presumably including (a) the viral glycoproteins on the outside surface and (b) anchorage and tegument proteins on the inside surface.

A central, puzzling issue is the mechanism of transport of viral glycoproteins to the nuclear membranes. A no less intriguing question involves the nature of the interaction of viral membrane proteins among themselves: Do they form well-defined structures, or do they float freely in the lipid bilayer?

To date, the viral membrane proteins identified in HSV-infected cells have been mostly glycosylated surface proteins. Analyses of the predicted structures of proteins based on the nucleotide sequence of open-reading frames suggest that the HSV-1 genome may specify other proteins that could traverse the lipid bilayer several times (e.g., the products of open-reading frames UL10, UL20, UL43, and UL53) (294).

Available evidence suggests that the general pattern of the biosynthesis of herpesvirus glycoproteins follows that of eukaryotic cell glycosylated proteins (45,474). Specifically, nonglycosylated precursors of herpesvirus membrane proteins are synthesized on polyribosomes bound to the rough endoplasmic reticulum. Glycosylation includes translational and post-translational events. Thus, *N*-glycosylation is initiated by transfer of preformed glycans [$(glucose)_3$-$(mannose)_9$-(N-acetylglucosamine)$_2$] from a dolichol phosphate lipid carrier to asparagine residues in the sequence Asn-X-Thr/Ser (X can be almost any amino acid) of a nascent polypeptide (238). During transit to the Golgi bodies, the oligosaccharide chains are trimmed by glucosidases and mannosidases to yield a glycan with five mannose and two *N*-acetylglucosamine residues. The high-mannose glycans are frequently converted by glycosyl transferases located in the *trans*-Golgi to complex glycans that consist of a pentasaccharide core [$(mannose)_3$-N-acetylglucosamine$)_2$] and a number of side chains (antennae) with the overall composition of sialic acid–galactose–*N*-acetylglucosamine. Fucose, when present, is usually added to the completed side chains (see ref. 238). O-linked glycosylation occurs less frequently than N-linked glycosylation (45,209,341,474). In some glycoproteins, notably gC of HSV-1 and gG of HSV-2, the O-linked oligosacharides are the major components (81,457); in other glycoproteins (e.g., gD of HSV-1 and HSV-2), however, the N- and O-linked oligosaccharide chains are present in roughly equal numbers (455). As is the case for many other mammalian glycoproteins (see ref. 25), O-linked glycosylation is initiated by the transfer of *N*-acetylgalactosamine to the hydroxyl group of threonine or serine and is followed by the addition of galactose and one or two sialic acids in the Golgi apparatus (81,456).

Current information on the structure of the HSV glycans has been summarized in detail elsewhere (45,474). Thus, all types of glycans (N-linked high-mannose; N-linked complex type; and O-linked) have been reported to exist in HSV-1 glycoproteins. HSV-1 glycoproteins are characterized by microheterogeneity in the glycans they carry, due to differences in the extent of chain processing and sialylation. Microheterogeneity has been shown even at a single *N*-glycosylation site (45,455). The first O-linked sugar, *N*-acetylgalactosamine, is added to the glycoproteins that carry N-linked oligosaccharides poorly processed by mannosidases, prior to their routing to the Golgi (454). Processing of HSV glycoproteins is carried out mainly by the host glycosylation machinery inasmuch as viral glycoproteins made in cells defective in some glycosylation enzymes reflect the defect (44,456). However, the available data do not exclude the possibility that the virus specifies one or more enzymes whose functions are similar to those of the host.

Nothing is known regarding the function and requirements for O-linked glycosylation of herpesvirus glycoproteins. Whereas virus particles containing high-mannose glycans are infectious, at least the initial step in N-linked glycosylation is required for infectivity inasmuch as blocking of N-linked glycosylation by tunicamycin blocks the accumulation of glycosylated proteins and of enveloped virus (352,359,360). Conversion of high-mannose glycans into complex-type glycans appears to be required for the egress of the virus from the infected cell (44,208,239,456). There is considerable evidence that after synthesis the viral glycoproteins are transported to the plasma membrane and can be found in cytoplasmic membranes of the cell. The viral glycoproteins in the cellular membranes are the targets of the immune response to the virus. Viral glycoproteins specified by genes resident in the environment of the cells mature and are transported faster than the proteins specified by genes resident in the viral genome and expressed during infection. The difference may simply reflect timing and intracellular traffic congestion. In cells expressing both a glycoprotein gene resident in the cellular genome and the corresponding gene resident in the viral genome, the former gene is expressed earlier; the glycosylation and transport of this protein does not compete with that of abundant viral glycoproteins made later from transcripts of genes resident in the viral genome (10).

Nothing is known of the mechanism by which viral glycoproteins enter nuclear membranes. As noted below, it has been suggested that virions enter the Golgi apparatus after envelopment. In polarized kid-

ney cells, viral glycoproteins are sorted to the basolateral membrane.

Several lines of evidence suggest that HSV membrane proteins form specific complexes. The existence of one complex, the Fc receptor formed by gI and gE, can be deduced from the observation that monoclonal antibody to either precipitates both glycoproteins (205). Another observation relates to an aberrant property of some mutants of HSV-1 and HSV-2 which cause infected cells to fuse.

Both HSV-1 and HSV-2 cause infected cells to round up and cling to each other. Some viral mutants cause cells to fuse into polykaryocytes; this fusion may be cell-type-specific or cell-type-independent (105,175, 434). Polykaryocytosis has been studied for several reasons: (a) as a probe in the structure and function of cellular membranes, reflected in the "social behavior of cells," (b) as a tool for analyses of the function of viral membrane proteins, and (c) as a model of the initial interaction between HSV and susceptible cells that results in the fusion of the viral envelope and the plasma membrane (45,407,408,474). Cell fusion induced by HSV requires full processing of high-mannose glycans to complex glycans up to the addition of sialic acid. In this instance it is not clear whether sialilated glycans must be present (a) only in viral glycoproteins located on the surface of the infected recruiter cells or (b) in the recruiter cells as well as in the uninfected cells to be recruited in polykaryocytes (45,474).

Polykaryocytosis can be viewed as an aberrant manifestation of the interaction of altered membrane domains of infected cells and unaltered membranes of juxtaposed cells (407,408). Genetic analyses have shown that mutations (*syn*) which confer the capacity to fuse cells map in at least four (and possibly more) loci within the viral genome (31,89,269,364,366,431, 439,548). Only one of these loci is within the domain of a viral glycoprotein (gB) and has been mapped in the carboxyl-terminal, cytoplasmic domain of the protein. One interpretation of this observation is as follows: The membrane proteins form complexes whose structure and conformation become altered by mutations in any of the component polypeptides, and the changes in conformation are similar to those which occur in the envelopes of virions interacting with the plasma membrane (434).

Envelopment

Nuclear DNA-containing capsids attach to patches of modified inner lamellae of the nuclear membrane and become enveloped in the process. The emphasis on "DNA-containing" capsids is a result of electron-microscopic observations which show that envelopment of empty capsids occurs rarely, although there is no evidence that "full" capsids contain a full-length molecule of HSV DNA (417). As discussed above, Vlazny et al. (513) demonstrated that capsids containing fragments of HSV DNA less than standard genome length are retained in the nucleus. A plausible explanation for this phenomenon rests on the observation that DNA-containing capsids differ from empty capsids with respect to protein VP22 (ICP35) (32,34,150). Conceivably, capsids become modified during packaging of the DNA, and only modified capsids are able to bind to the underside of the thickened patches containing viral proteins in the nuclear membranes.

There is general agreement that the inner lamellae are the site of initial envelopment (Fig. 5). However, even cursory examinations of thin sections of infected cells elicits the rediscovery that envelopment occurs in the cytoplasm, since the cytoplasm contains capsids juxtaposed to patches of modified cytoplasmic membranes in the process or envelopment or de-envelopment. Stackpole (479) is the originator of the idea that the capsids become enveloped at the inner lamellae, de-enveloped at the outer lamellae, re-enveloped by the endoplasmic reticulum, and released in the extracellular environment either by envelopment at the plasma membrane or by fusion of vesicles carrying enveloped virus at the plasma membrane. The large number of particles seemingly undergoing cytoplasmic envelopment makes a strong case for this model. However, thin sections showing capsids being enveloped at the nuclear membranes are extremely rare, suggesting that the process of envelopment is very rapid. Since every capsid undergoing putative envelopment in the cytoplasm must have been enveloped and de-enveloped in transit through the nuclear membranes, the disparity in the numbers of capsids being enveloped at the nuclear and cytoplasmic membranes suggest that either (a) the rate of envelopment at the nuclear membranes is significantly faster than that at the cytoplasmic membranes or (b) the capsids in juxtaposition to cytoplasmic membranes are artifacts and represent capsids that are de-enveloped or arrested in their movement through membranes. The key question of whether the cytoplasmic, semienveloped capsids are in fact in the process of being enveloped (rather than being arrested, transient structures resulting from de-envelopment) remains unanswered. One hypothesis for the presence of partially enveloped structures at cytoplasmic membranes is that virions contained in the endoplasmic reticulum attach to receptors and reinfect the cell from within, but the capsid is not transported to the nuclear pore for lack of cytoskeletal connections. It is of interest that cytoplasmic semienveloped capsids are prevalent in continuous cell lines but

are less prevalent in infected primary human diploid cells.

Transit Through Cytoplasm; Egress and Re-entry

In the cytoplasm, intact enveloped particles are usually seen inside structures bounded by membranes (85,448). This observation is not unpredicted, inasmuch as structures bounded by membranes with surface glycoproteins are not likely to fare well unprotected in the cytoplasm. In two-dimensional sections, these structures appear to be vesicles. These vesicles may well represent the vehicles by which virions transit through the cytoplasm. In a few electron micrographs, tubular structures have been seen, and the probability exists that in some cells the cisternae of the endoplasmic reticulum extend to the plasma membrane (448). Any model for cytoplasmic transit of virions that culminates in egress must take into account that (i) all conditions which lead to a block in glycoprotein maturation hinder virus egress and induce an intracytoplasmic accumulation of enveloped particles containing immature glycoproteins (45,239,474), and (ii) nuclear membranes appear to contain predominantly immature forms of glycoproteins (67). It has been suggested that virus egress is achieved by a mechanism akin to reverse phagocytosis, a term which conveys the direction (rather than the mechanics) of the motion. Thus, Johnson and Spear (208) proposed, in part on the basis of studies done with monensin, that virions are secreted via the Golgi apparatus following a pathway similar to that taken by secreted soluble proteins.

In cells infected with a mutant virus containing a lesion mapping in the S component, but not in cells infected with wild-type virus, empty capsids accumulate in large numbers at the outer surface of the nuclear membrane, suggesting the possibility that HSV encodes a function to prevent reinfection of cells, particularly with virus which had been released from these cells (505). This function has been attributed to glycoprotein D (43).

Membrane Proteins

In addition to the glycycoproteins listed earlier in the text, several other membrane proteins have been postulated to exist on the basis of mapping of *syn* mutants described below. Furthermore, analyses of the structure of putative proteins predicted to exist on the basis of the HSV-1 DNA sequence (294) have identified several proteins whose predicted structures are consistent with those of multimembrane spanning proteins. These are the proteins predicted to be encoded in the open-reading frames UL10, UL20, UL43, and UL53. Other proteins with the potential to interact with membranes are UL34, UL45, and US9 (294).

REGULATION OF VIRAL GENE EXPRESSION

Structure of HSV mRNAs

The properties of viral mRNAs are central to a discussion of the regulation of viral gene expression. HSV DNA is transcribed by RNA polymerase II (75). Viral mRNAs are capped, methylated, and polyadenylated, although nonpolyadenylated RNAs of the same sequences can be isolated (13,18,19,467,469,490). Internal methylation is readily apparent in RNA made early, but not late, in infection (19). Notwithstanding the efficient expression of HSV genes in the environment of higher eukaryotic cells, only a relatively small proportion of HSV mRNAs are derived by splicing. Genes sharing 5′ (or, particularly, 3′) termini have been described (310,515). Attention has also been drawn to (a) multiple initiation sites for the transcription of selected HSV genes (139,330,459,522,549) and (b) the occasional RNAs that extend beyond the usual polyadenylation site (6,178,394). In contrast with the orderly transcription of intact cells are the random initiations experienced by more than one laboratory in nuclear run-off transcription assays late in infection (151,528). The abundance and stability of the various HSV-1 mRNAs appear to vary (136,137,467,469). In general, mRNAs of α and β genes appear more stable than those of γ genes (544). Viral mRNAs may persist in the cell after their translation ceases (210,241). Although complementary RNA sequences are readily detected in infected cells, double-strand RNA does not accumulate (197, 242).

The Environment of the Viral Genes

The open-reading frames identified to date are embedded, for the most part, in domains exhibiting both virus/host-common (e.g., TATAA boxes) and virus-specific 5′ sequences. The absence of TATAA boxes from some transcriptional units has been noted (see, e.g., ref. 57).

Studies on the structure of HSV genes have focused on two specific objectives. The first objective involved (a) the minimal promoter domain and (b) the *cis*-acting sites required for gene expression. The second objective was to identify the *cis*-acting sites that confer upon the target gene the capacity to be regulated as an authentic viral gene. Only a few HSV genes have been analyzed in sufficient detail to reveal and identify the *cis*-acting signals embedded in them. The most thoroughly studied, and the one that has generated the most conflicting results, is the *tk* gene. Also worthy of

discussion are the α4 gene (Fig. 9) and, to a much lesser extent, two representative γ_2 genes, although the level of controversy is not nearly as high.

In the α4 gene, the 5′ nontranscribed domain extending upstream from the cap site to nucleotide −110 is capable of imparting to a reporter gene the capacity to be transcribed efficiently in the absence of viral *trans*-activating factors (279,280,367). Other than the transcription initiation site, the *cis*-acting sites that affect expression have not been investigated in detail. The sequences upstream from nucleotide −110 confer on α and β promoters a higher basal level of expression as well as the capacity to be induced as α genes by viral *trans*-activating factors (245). The higher level of expression conferred by the sequences upstream from nucleotide −110 is very likely due to the SP1-binding sites embedded in G+C-rich inverted repeats which abound in that region (214,215,245,278–280). At least one sequence that confers inducibility as an α gene is the *cis*-acting site for αTIF [5′ NC GyATGn-TAATGArATTCyTTGnGGG 3′ (245,279)]. Separation of promoter and regulatory domains has been noted in other α genes. Although G+C-rich stretches are frequent, SP1-binding sites have not been reported in other α genes (Fig. 9).

ICP4-binding sites have been reported in the promoter-regulatory domain and across the transcription initiation site of the α4 gene, as well as in the promoter domain of the α0 gene, but not in the other α genes (120,246,247,328).

The initial analyses of the *tk* gene rested on the erroneous notion that it exemplified the structure of a typical eukaryotic gene. Only recently has there been an effort to understand its structure as a viral, β gene. Several features are of particular interest. The *tk* gene appears to have two transcription initiation sites, and mRNAs derived from both sites have been reported (98,375,549). The role of these sites in the expression of the gene in lytic infection is not clear. The 5′ nontranscribed region has been very thoroughly investigated by McKnight and co-workers (104,305–309) and by Silverstein and co-workers (108,109,142). The initial studies identified a 110-nucleotide region upstream from the site of initiation of transcription that was minimally required for efficient expression in the absence of viral *trans*-activating factors. This promoter contains a "proximal" *cis*-acting site and two "distal" sites, all of which are important for constitutive expression of the *tk* gene and probably function as punctuation sites and sites for binding of RNA polymerase and accessory factors (549). In subsequent studies a CCAAT box, two SP1-binding sites (214), and an octamer motif (ATTGCAT) upstream of −116 were also identified (350). Mutations in all of these sites affect expression of the uninduced gene. However, attempts to define a *cis*-acting site that is virus-specific have not been successful. The protocol employed involved studies of linker scanning mutations. Inasmuch as the authors failed to identify mutations which affected *trans*-activation but not expression in the absence of viral *trans*-activating factors, they concluded that the viral factors which *trans*-activate viral β gene

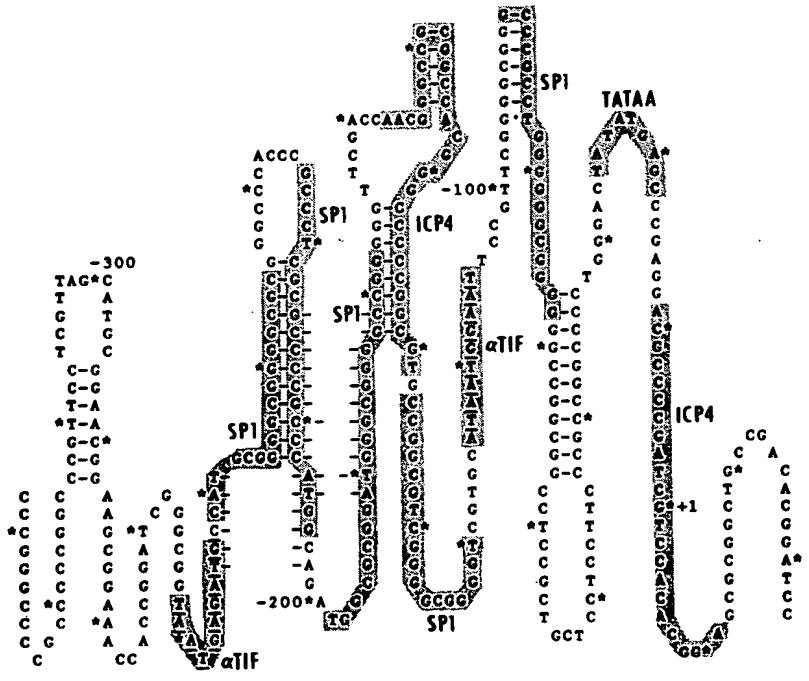

FIG. 9. Schematic representation of the structure of the 5′ nontranscribed domain of the α4 gene. An asterisk marks every 10th nucleotide. The transcription initiates at nucleotide +1. The shaded areas represent the binding sites of TATAA protein, SP1 and ICP4, and the host protein–αTIF complex. The sequence is shown in a folded form to emphasize the presence of the perfect G+C-rich inverted repeats that abound in this domain of the gene. In some instances, the G+C-rich regions can form alternate stem structures; these are identified by the dashes. (Data were taken from ref. 280.)

expression act on a host factor and not directly on DNA.

More recent studies have focused on the interaction of ICP4 with domains of the *tk* gene and on mutations downstream from the *tk* cap site. Notwithstanding earlier reports that ICP4 does not bind to the *tk* gene directly (97,403), the following facts are evident: The ICP4 does indeed bind to several domains of the gene both upstream and downstream from the cap site, and the binding sites include both (a) sequences similar to the consensus binding site reported by Faber and Wilcox (119) and (b) highly degenerate sites with little resemblance to the consensus (e.g., see ref. 316). Of particular interest are (a) the ICP4-binding sites in the transcribed noncoding domains and (b) the role of the transcribed noncoding sequences with respect to the regulation of *tk* as a β gene, as discussed below.

The structure of the γ genes, and particularly of the γ_2 genes, is the least well understood and is likely to be highly variable. Analyses of three genes—glycoprotein C, UL49, and US11—suggest that the sequences required for efficient expression include the TATAA box and extend into the 5′ transcribed noncoding domain (73,98,118,180,181,211–213,289,290, 458,466). The sequences required for the γ_2 regulation of these genes appear to also include sequences downstream from the TATAA box. Embedded in transcribed domains, as well as in the 5′ nontranscribed sequences, are ICP4-binding sites whose functions are not yet known (316).

Regulation of HSV Gene Expression: *trans*-Acting Factors

Given the complexity of herpesvirus multiplication, the reproducibility of the general pattern of virus multiplication long ago suggested that the events must be tightly regulated (413). Regulation of HSV gene expression has become a major focus of research, and the mechanism by which the virus regulates its replication remains an exciting area of investigation.

Turning on HSV genes involves several elements: (a) *cis*-acting sites for both viral *trans*-acting factors and cellular transcriptional proteins, (b) *trans*-acting signal proteins specified by the virus, and (c) both viral and cellular factors involved in viral DNA synthesis and in posttranslational modification of viral proteins. It is convenient to review the features of the cascade regulation of gene expression illustrated in Fig. 4 and described above by considering the *trans*-acting factors and their *cis*-acting sites.

α-*Trans*-Induction Factor (αTIF)

α genes are induced by a structural protein of the virus, αTIF. The *cis*-acting site (245,280)

5′ NC GyATGnTAATGArATTCyTTGnGGG 3′

binds a host protein (248,249) designated variously as OTF-1, NFIII, or αH1 (248,249,342). αTIF is packaged in 500–1,000 copies per viral particle (170). It does not interact with the viral DNA directly; rather, it binds to the complex formed by the host protein and the *cis*-acting site on viral DNA (145,303,336,374,507). The *trans*-activating function of the protein maps near its carboxyl terminus (438,506); its association with the DNA either as a chimeric DNA-binding protein or through αH1/OTF-1/NFIII, etc., is required for *trans*-activation. A characteristic of αTIF is that it is packaged in the virion tegument, and upon release of the intravirionic contents in the newly infected cell it is translocated to the nucleus independently of the viral DNA; that is, at nonpermissive temperature the mutant HSV-1(HFEM)*ts*B7 *trans*-activates α-regulated genes even though the viral DNA is not released from capsids (21).

The induction of α genes by αTIF presents numerous unresolved puzzles. Foremost, there is no explanation for the failure of the α genes to be expressed late in infection in light of the massive amounts of αTIF accumulating in the infected cells at that time. Exhaustion of functional host protein is a possibility, but the presence of OTF1/NFIII/αH1 capable of binding to DNA can be readily demonstrated in infected cells late in infection.

The second puzzle stems from the observation that the footprint of the host protein required for αTIF binding is in the sequence ATGnTAAT. The 5′ domain of this sequence is the least conserved domain of the *cis*-acting site, whereas the sequence TAATGArAT is highly conserved (280), suggesting either that αTIF makes contact with the DNA in the GArAT sequence or that another DNA-binding protein is also involved.

ICPO

The product of the α0 gene is predicted to be 80,000 in apparent molecular weight, but in denaturing polyacrylamide gels it migrates with an apparent molecular weight of 110,000–124,000, depending on the type of cross-linking agent used and on the acrylamide concentration. $\alpha 0^-$ mutants are viable in cell culture, and *ts* mutants have not been reported (437,489). In transient expression systems, ICPO has been reported to promiscuously *trans*-activate transfected genes by itself or in combination with ICP4 (114,117,142–144,337–339,358,391,440). Of the various experimental designs, the most convincing are those in which the *trans*-activation of target genes was done in conjunction with ICP4. The function of ICPO in infected cells is not known and is not readily apparent. The transient expression studies suggest that it may act as a *trans*-activator of the α4 gene. No *cis*-acting site is readily

apparent. Deletion mutants in the α0 gene grow in cell culture, albeit more sluggishly than the wild-type virus, particularly at low multiplicities of infection (437,489).

ICP4

This protein is predicted to be 132,835 in translated molecular weight (294). Most fresh HSV-1 isolates show *ts* α4 activity, and 37°C/39°C ratios of plating efficiencies as high as 10^6 have been recorded [e.g., HSV-1(F)]. It is then perhaps not surprising that *ts* mutants in the α4 gene have been readily isolated by a number of laboratories (371,372,521). ICP4 forms three bands (designated as 4a, 4b, and 4c) in denaturing polyacrylamide gels (357). In cells infected with wild-type virus, the fastest migrating band (band 4a) has an apparent molecular weight of 160,000 and is readily detected in the cytoplasm after pulse-labeling with radioactive precursors (183,184,357,539). It is also the only form accumulating in cells infected with certain α4 *ts* mutants and incubated at nonpermissive temperatures (233). Bands 4b and 4c have apparent molecular weights of 163,000 and 170,000, respectively, and accumulate in the nucleus (357,539). The accumulation of the slower migrating bands coincides with (a) translocation of the protein into the nucleus and (b) labeling with inorganic ^{32}P phosphate added to the medium (124,357). ICP4a and ICP4c can be pulse-labeled with ^{32}P during the reproductive cycle long after the synthesis of this protein ceases, suggesting that phosphate cycles during infection (539). Preston and Notarianni (376) reported that α4 accepts ADP ribosylation in isolated nuclei; more recent studies (J. Blaho, N. Michael, and B. Roizman, *manuscript in preparation*) indicate that ICP4 is poly(ADP)-ribosylated. ADP ribosylation could account for the labeling of ICP4 by inorganic phosphate from the medium (357), but phosphate also cycles on and off ICP22 and ICP27 (539); whereas, ICP4 appears to be the only α protein to be poly(ADP)-ribosylated.

ICP4 is the major *trans*-activator of HSV genes. Long a subject of study, it has generated a rich, passionate (and sometimes confusing) literature. $\alpha4^-$ mutants grow only in cells expressing ICP4 proteins from a copy of the α4 gene embedded in the cellular genome. Fine anatomical dissection of the gene has outlined domains necessary for autoregulation of α genes, for the synthesis of proteins made later in infection, for phosphorylation, and for nuclear transport (95–97,189,351,471). It is convenient to consider the effects of ICP4 on α, β, and γ genes separately.

In the case of *ts* α4 mutants, both copies of the gene are mutated, as would be expected for the expression of the *ts* phenotype (233). The phenotypes of these mutants vary. At the nonpermissive temperature, some mutants express both α proteins and selected sets of proteins normally made later in infection (93). A most interesting group of *ts* mutants in the α4 gene overproduce α proteins at the nonpermissive temperature (93, 521). There is convincing evidence that ICP4 turns off its own synthesis and that this autoregulation correlates with the binding of the protein to a *cis*-acting site across the transcription initiation site of the gene (97,328,403). Comparisons of the α RNAs accumulating in cells infected and maintained at permissive and nonpermissive temperatures indicate that the α genes subjected to repression are primarily α4 and α0 (J. Hubenthal-Voss and B. Roizman, *unpublished studies*). These genes are the only ones in which a high-affinity ICP4-binding site corresponding to the consensus sequence ATCGTCnnnnCnGnn have been reported (246,247,328). As noted above, in the case of the α4 gene, the binding site ATCGTCcacaCgGag is across the cap site (328). In the case of the α0 gene, the binding site ATCGTCactgCcGcc is at position −64 to −49 (247). The correlation between binding activity of ICP4 to DNA and shut-off of α4 transcription, if confirmed in the case of α0 as well, would suggest that ICP4 can turn off transcription even when the binding site is upstream of the transcription initiation site.

The *trans*-activation of β genes has been discussed above. ICP4-dependent activation of transcription of a β gene embedded in the viral genome occurs from a very much lower level of basal expression than that seen from an isolated gene introduced into the same cells. After *trans*-activation, the level of *tk* gene expression is higher than that attained in cells transfected with the isolated *tk* gene. ICP4 DNA-binding sites in the domain of the *tk* gene both upstream of the cap site and downstream from nucleotide +50 have been demonstrated by several groups (246; Mavromara-Nazos and Roizman, *unpublished studies;* S. Silverstein, *personal communication*) but not by others (403). Studies by Halpern and Smiley (163) and by Mavromara-Nazos and Roizman (290) have failed to demonstrate a significant role of the binding to sites downstream of nucleotide +51.

It would seem to us that *trans*-activation of β genes involves two functions: (i) release from a repressive state and (ii) *trans*-activation. Since neither of these occurs at the nonpermissive temperature in cells infected with *ts* mutants in the α4 gene, then at least one, the initial event, depends on ICP4. To aficionados of transient expression it is worthwhile to point out that in cells transfected and selected for *tk* activity, the ratio of induced to basal TK activity after *trans*-activation with virus is considerably lower than that obtained in cells which are (a) transfected with a plasmid containing TK and another marker and (b) selected for the other, covalently linked marker (245). We interpret this to indicate the following: For a constant ratio of *tk*

genes per cell, the fraction of derepressed *tk* genes is higher in the cells selected for TK activity, but α4 derepresses *tk* genes in both systems. Inasmuch as binding of ICP4 to DNA correlates with repression irrespective of the position of the binding site, it may be useful to entertain the possibility that derepression may involve modification of a host protein rather than binding to DNA per se.

Earlier in the text we noted that as in the case of the β-*tk* gene, the γ genes are expressed and regulated differently in the context of the environment of the viral genome than in that of the cellular genome. The assessment of the role of ICP4 in the *trans*-activation of γ genes is complicated by several additional factors. Foremost, γ genes and especially γ_2 expression require viral DNA synthesis as a *cis*-acting function (289). Second, although it has been reported that the cap site and TATA box of the gene encoding US11 is all that is required for "fully efficient regulated activity" (211), the regulatory domains of at least two γ_2 genes appear to be downstream from the TATAA box and very likely include the 5′ transcribed noncoding domains (290). These domains contain ICP4-binding sites (316; P. Mavromara-Nazos and B. Roizman, *unpublished studies*). A possible role of α27 has also been noted, as described below.

Recently, two observations suggested a possible scenario for viral gene regulation: First, a chimeric gene consisting of 5′ nontranscribed domains of the β-*tk* gene and the 5′ transcribed noncoding domains of a γ_2 gene was expressed both early and late in infection (recombinant R3820), while a chimeric gene consisting of γ_2 5′ nontranscribed sequences and β-*tk* 5′ transcribed noncoding domains was barely expressed (recombinant R3821) (290). In addition, Arsenakis et al. (9) reported that the *gD* gene contained in a large HSV-1 DNA fragment was expressed in baby hamster kidney cells lacking the α4 gene but was not expressed in cells expressing significant amounts of ICP4 protein. One hypothesis (289) of at least heuristic value is that (i) the *trans*-activation of γ_2 genes requires removal, during DNA synthesis, of a transcription blocking factor and also requires *trans*-activation of transcription by another factor, (ii) the transcription blocking factor is effective only for transcriptional *trans*-activators operating downstream from the block and does not affect transcriptional activators acting upstream from the block, and (iii) the *cis*-acting sites for both the blocking factor and the transcriptional factor are in the 5′ transcribed noncoding domains. This hypothesis is consistent with the observations that (i) 5′ transcribed noncoding domains contain the *cis*-acting sites necessary for γ_2 gene expression, (ii) genes whose 5′ domains consist of the *tk* 5′ nontranscribed domains fused to the γ_2 5′ transcribed noncoding domains are regulated as both β and γ_2 genes, and (iii) in cells infected with $\alpha 27^-$ mutants, viral DNA is made but γ_2 genes are not expressed (i.,e. DNA synthesis alone is not enough to activate γ_2 gene expression). Does ICP4 block transcription, *trans*-activate it, or both? The evidence that, in cell-free systems, ICP4 increased the transcription of *gD* [a γ_1 gene (499)] is not in itself impressive, since (i) the ICP4-binding site tested was upstream from the reported minimal sequence required by gD to be regulated as a γ_1 gene [nucleotide −55; (113,115)], (ii) in transient expression systems, for what it is worth, late γ_2 gene expression was activated at low concentrations of the ICP4 gene but not by high concentrations of the gene, and (iii) the amount of ICP4 used in that study was arbitrary, without relevance to either known positive or negative *trans*-activation.

The hypothesis that ICP4 does not act directly on the DNA cannot be dismissed out of hand. Much has been made of the observation that α4 and the equivalent gene product of pseudorabies virus induce not only herpesvirus genes but also adenovirus and cellular genes (e.g., β-globin) introduced into cells by transfection (26,121,161). More recent studies have shown that the *trans*-activation of adenovirus late gene expression by the ICP4 equivalent of pseudorabies virus is through enhanced formation of transcription initiation complexes (1). While very revealing of a biased experimental design (the experimental design assumed *a priori* that the herpesvirus major regulatory protein *trans*-activates in a nonspecific fashion, since it is hardly likely that herpesvirus *trans*-activating proteins evolved to *trans*-activate adenovirus genes), these studies in fact do not contradict the evidence that ICP4 acts directly by binding to specific sites on the DNA. ICP4 appears to be a multifunctional protein. It could very well be that ICP4 and its homologues derive from a cellular transcriptional factor. Foremost, however, ICP4 has a dual function as a repressor and as a *trans*-activator; the function of the protein may well be determined by the position of its binding site, the strength of its binding to the DNA, and the nature of the posttranslational modification to which it has been subjected (316).

Other Factors Implicated in trans*-Activation of Viral Genes*

ICP8, ICP22, and ICP27 have been implicated in gene regulation.

The molecular properties of ICP8 were described earlier in the text. Most (but not all) of the effects of ICP8—namely, enablement of late gene expression and the effect of mutants on the expression of early genes—could be rationalized from its role in late DNA synthesis, but only because its role in DNA synthesis is known. To explain the shut-off of expression of viral

genes, it is necessary to postulate occlusion of transcription by overreactive ICP8 (151–153).

The predicted translated and apparent molecular weights of ICP22 are 46,521 and 72,000, respectively (183,184,294). Studies on a deletion mutant (368) indicate that the α22 gene is required in some cell lines but not in HEp-2 or Vero cells, presumably because the latter two cell lines express functions similar to that of α22 (450). In the nonpermissive cells (rodent cell lines and human cell strains), viral DNA is made, but late (γ_2) genes are not expressed efficiently.

The predicted apparent molecular weights of ICP27 are 55,249 and 58,000, respectively (183,184,294). Both *ts* and deletion mutants in the α27 gene have been reported (291,436). Notwithstanding a report to the contrary (281), the gene appears to be essential; cells infected with deletion mutants synthesize viral DNA, but late (γ_2) genes are not expressed (291; D. Knipe, *personal communication*). The phenotype of $\alpha27^-$ viruses resembles that of mutants in the β8 gene (i.e., reduced synthesis of viral DNA, absence or reduced synthesis of γ proteins, and increased synthesis of β proteins). In transient expression systems, ICP27 has both positive and negative effects (116,376a,396,453,491a).

Posttranscriptional Regulation

The evidence for posttranscriptional controls is based on reports that translocation of viral transcripts into the cytoplasm appears to be regulated (216,218,241). Specifically, the genetic complexity of the RNA accumulating in the nuclei of cells infected with HSV in the presence of cycloheximide and maintained in medium containing the drug was greater than that observed in the cytoplasm. In retrospect, the interpretation of the data is not clear. The failure to demonstrate RNA complementary to β genes (e.g., *tk*) in nuclei of infected cells treated with cycloheximide (266) suggests that the transcripts accumulating in the nuclei might be random transcripts of the DNA rather than transcripts of specific genes belonging to the β and γ groups.

The evidence for translational regulation is based on several observations. Specifically, the inhibition of host protein synthesis by structural components of the virion soon after infection (125,333,395) and the inhibition of α gene product synthesis by subsequent gene expression (124,184) are translational events inasmuch as they occur in physically and chemically enucleated cells. A significant finding to emerge from the studies by Kwong and Frenkel (252) and Oroskar and Read (344) is that virion structural components exert an inhibitory effect on both host and α protein synthesis, inasmuch as mutants defective in the virion host shutoff function produce more α gene products than do their wild-type parents. It has been suggested that mRNAs of genes turned off in the transition from α to β to γ gene expression remain associated with polyribosomes. Studies by Johnson and Spear (210) reported the continued cytoplasmic accumulation of functional mRNA specifying glycoprotein D, a γ_1 polypeptide, after gD synthesis had declined.

HSV Gene Regulation: The Problems in Experimental Designs

In the preceding section we reported the conclusions that are supported by evidence. Examination of the literature of the past several years has disclosed three problems. Central to the evaluation of the available data are three highly significant, inescapable (and unfortunately inflammatory) issues.

The first is the evaluation of the methodology on which much of the data rests. The gold standard for the studies of viral gene regulation is the pattern of expression in productive infection of natural or reporter genes contained in the viral genome. Tests of modified *trans*- or *cis*-acting domains of individual genes are easier to perform and may, in some cases, be more meaningful if they can be done in the environment of the cell and in the presence of only a minimal amount of viral genetic information. However, the validity of such tests hinges on the extent to which they reproduce the regulation of the gene embedded in the viral genome and expressed in the course of viral infection. The expression of isolated α genes, in biochemically transformed cells or in transient expression systems, appears to mimic, to some extent, the regulation of the corresponding genes contained in viral genomes during productive infections (367). Notwithstanding the massive number of transfections which argue that ICP0 is a promiscuous *trans*-activator, supporting evidence from studies on deletion mutants in the α0 gene is not readily available. The transfection system apparently fails if more than two components of the regulatory pathway are introduced into the cell simultaneously—for example, the cotransfection of αTIF, α4, and the intended target gene of α4 (403). In the case of γ_2 genes, the transient expression system yields totally false results: Viral genes permanently integrated in cellular genomes or transiently expressed after transfection are regulated as β genes (10,28,466). The transfection system has given rise to a veritable cottage industry, but the results it has generated are not totally reliable. What is the evidence that viral genes other than those carrying the α-*cis*-acting sites can be regulated in that system in a mode which resembles viral gene regulation? If γ_2 genes are regulated as β genes, then what is the evidence that β genes in transfected cells are regulated as bonafide β genes?

The second issue centers on distinctions between experimental objectives and experimental design. It is intuitive that experimental designs should discriminate between alternative hypotheses and be able to predict a specific outcome that would unambiguously reject untenable alternatives. However, consider the following situation. As noted earlier in the text, the *tk* gene has been extensively used in transient expression systems for analyses of its *cis*-acting domains, and the gene is readily expressed in such systems. This is not the case for the *tk* gene contained in the viral genome and introduced into cells by infection. In this instance, in the absence of competent α proteins, the *tk* gene is not expressed. In the past several years a score of laboratories have introduced mutations into the viral genome in an attempt to define the *cis*-acting site for *trans*-activation of the *tk* gene by ICP4; not once has it been stated *a priori* what should be the phenotype of a *tk* gene whose ICP4 *cis*-acting site has been inactivated without also reducing the ability of the uninduced gene to be expressed! The unstated assumption is as follows: The ICP4 *cis*-acting site is distinct from that of the essential promoter sites, and in the absence of the *cis*-acting site for induction by ICP4 the *tk* gene would be expressed, albeit poorly. But how can this be true if in the absence of ICP4 the inherent capacity of the *tk* gene to be expressed is blocked? If viral proteins inhibit expression that is unblocked by ICP4, the only expected effect of mutations is full *tk* expression except when *cis*-acting sites required by the uninduced promoter are inactivated.

An additional point that seems to be ignored is that the *tk* gene is a late β gene; that is, its expression concurs with, rather than precedes, DNA synthesis as compared with ICP6 (the major component of ribonucleotide reductase) or ICP8 (the single-strand DNA-binding protein), which are abundantly expressed early in infection. In cells infected with wild-type virus, the expression of the *tk* gene is dependent solely on the parental genome, and progeny DNA does not appear to contribute significantly to the TK pool. Quite to the contrary, because the expression of β genes is reduced late in infection, inhibition of DNA synthesis enables larger amounts of TK to accumulate in the infected cells.

It would appear that the existing data do support the hypothesis that ICP4 *trans*-activates the *tk* gene by augmenting the effect of a host transcriptional factor, and therefore its *cis*-acting site is that of the host factor. However, the data also support the hypothesis that ICP4 merely releases the *tk* gene from a repressed state. In fact, experimental designs employed to date may also support additional hypotheses.

The last issue concerns the definition of the elements that play a role in the regulation of gene expression. In principle, we need to differentiate between two elements: (i) proteins whose function is to *trans*-activate one or more viral genes by acting on specific *cis*-acting sites and (ii) viral proteins which affect viral gene expression by a global action on the viral genome. αTIF and ICP4 are examples of bonafide regulatory proteins acting in *trans* on specific *cis*-acting sites. Clear examples of proteins which alter regulation by global effects on viral genomes are the single-strand DNA-binding protein and DNA polymerase. Malfunction of either protein (e.g., DNA$^-$ *ts* mutants at the nonpermissive temperature) would block the appearance of γ_2 proteins. In the case of the single-strand DNA-binding protein and the polymerase, the global effects can be readily substantiated by showing that they are part of the minimal set of genes required for amplification of viral DNA through its origin of DNA synthesis. We should note, however, that ICP8 has been implicated in many more regulatory events than can be ascribed to DNA synthesis. These effects could be due to nonspecific occlusion of transcription by overactive binding of ICP8 to single-strand DNA. The less clear situation concerns genes whose functions are not known and which affect viral gene expression. They may be bonafide regulatory proteins which affect transcription by acting on specific sites at or near the genes they *trans*-activate, or they may act globally in a manner which cannot be tested currently. The cases in point are the functions of the genes α27 and α22. Neither protein has been unambiguously shown to bind to viral DNA. Deletion mutants have shown that both genes affect late gene expression, selectively in some cells in the case of α22$^-$ and in all cells tested in the case of α27$^-$ viruses. Are they site-specific *trans*-activators, or are they proteins with global effects on the structure of the viral genome?

THE FATE OF THE INFECTED CELL

Cells productively infected with herpesviruses do not survive. Almost from the beginning of the reproductive cycle the infected cells undergo major structural and biochemical alterations that ultimately result in their destruction.

Structural Alterations

Changes in Host Chromatin

As described in detail elsewhere (410,417) and shown in Fig. 10, one of the earliest manifestations of productive infection is in the nucleolus; it becomes enlarged, becomes displaced toward the nuclear membrane, and ultimately disaggregates or fragments. Concurrently, host chromosomes become marginated, and later in infection the nucleus becomes distorted and

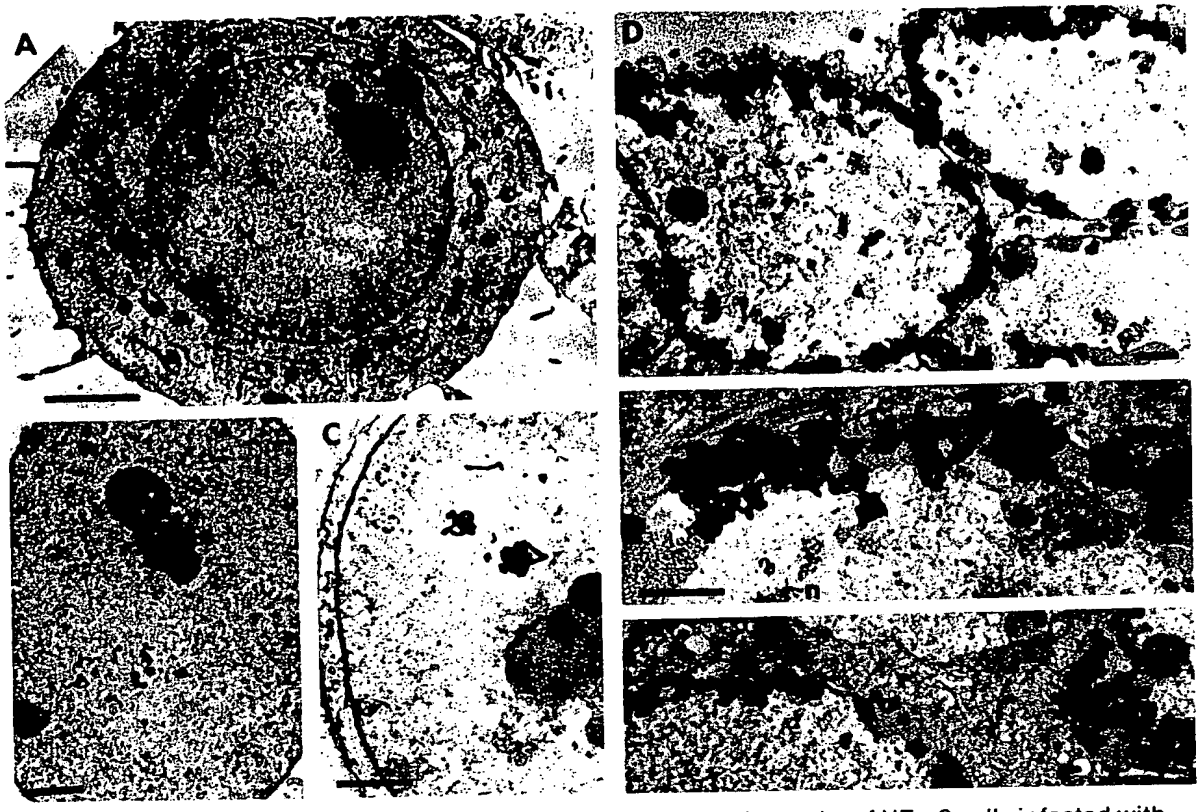

FIG. 10. Electron photomicrographs of thin-section autoradiography of HEp-2 cells infected with herpes simplex virus. **A:** A 4-hr-infected cell pulse-labeled for 15 min with [^{3}H]methyl thymidine prior to fixation. **B** and **C:** Enlargements of nuclei prepared as in part A. Note the disaggregated nucleolus. **D:** Portions of three nuclei of 18-hr-infected cells labeled with [^{3}H]methyl thymidine prior to infection. Unlabeled thymidine was present in the medium during and after infection. **E** and **F:** Electron micrographs of nuclei taken at higher magnifications. Note the aggregation of chromatin at the nuclear membrane. One of the cells in parts D and F did not synthesize DNA during the short labeling pulse. Abbreviations: n, nucleus; c, cytoplasm; v, aggregation of virus-specific, electron-opaque material. (From ref. 410 and from J. Schwartz and B. Roizman, *unpublished photomicrographs.*)

multilobed. The numerous protrusions and distortions have previously been mistaken for amitotic division (219,449). Margination of the chromosomes may or may not be linked with the chromosome breakage reported by numerous investigators (for review, see ref. 417).

Virus-Induced Alteration of Cellular Membranes

Changes in the appearance of cellular membranes (and, in particular, of nuclear membranes) are characteristic of cells late in infection. Deposition of material (tegument proteins?) on the inner surface facing the nucleoplasm or cytoplasm, but not in the space between inner and outer lamellae or cisternae of the endoplasmic reticulum, results in the formation of thickened patches along the membranes. Ultimately, the patches in the nuclear membrane coalesce and fold upon themselves to give the impression of reduplicated membranes (Fig. 5) (69,263,293,321,332,447,464).

The first inkling that herpesviruses modify cellular membranes was based on the observations that mutants differ from wild-type strains with respect to their effects on cells: Whereas wild-type viruses usually cause cells to round up and clump together, mutants may cause cells to fuse into polykaryocytes (105,407,408). These observations led to the prediction that herpesviruses alter the structure and antigenicity of cellular membranes—a prediction fulfilled by (a) the demonstration of altered structure and antigenic specificity (402,425) and (b) the presence of viral glycoproteins in the cytoplasmic and plasma membranes of infected cells (171,423,424,476).

The presence of gD in the plasma membrane of infected cells seems justifiable in light of the evidence cited earlier in the text, that gD precludes reinfection of cells with the progeny virus released from that cell.

gE and gI act as an Fc receptor (22,205,345,346). The function of the Fc receptor is not known. Several reports (60,138,452,470) indicate that gC binds the third component of complement, possibly in order to reduce the availability of the component for the immune lysis of the infected cell. Our knowledge regarding the processing, metabolism, and function of viral glycoproteins is not sufficient to deduce the reason for the presence of viral glycoproteins on the surface of the infected cell. The reason could be chance (a reflection of the natural flow of membrane-bound proteins) or design (the evolution of a pathway for cell-to-cell transmission of virus shielded from neutralizing antibody). Irrespective of the reason, the infected cell presents an altered antigenic specificity and becomes a target for destruction by the immune system.

The mutations (*syn*) which change the nature of the interaction of plasma membranes and affect the "social behavior of infected cells" have been mapped to several genetically unrelated locations on the HSV genome. Only one of these locations appears to map within the domain of a glycoprotein (gB) (282,434). Two other *syn* loci map within the UL24 and UL53 open-reading frames (89,196,364,366,434). A fourth locus has been mapped at the left terminus of the L component (UL1?) (269). All three predicted proteins contain hydrophobic regions suggestive of membrane proteins (294). This multiplicity of genes which affect cell fusion suggests that viral membrane proteins form interactive complexes and that alteration of any one component may alter the structure and function of the entire complex (434).

Host Macromolecular Metabolism

A characteristic of herpesvirus-infected cells is the rapid shut-off of host macromolecular metabolism early in infection. Thus, host DNA synthesis is shut off (Fig. 10) (421), host protein synthesis declines very rapidly, (252,395,414,494,495), host ribosomal RNA synthesis is reduced (518), and glycosylation of host proteins ceases (476).

HSV host shut-off occurs in two stages. The first stage, documented initially by Fenwick and Walker (125) and by Nishioka and Silverstein (333–335), involves structural proteins of the virus and does not require *de novo* protein synthesis. Thus, HSV shuts off host protein synthesis in physically or chemically enucleated cells (124); the shut-off was effected by density-gradient-purified virus but not by purified virus inactivated by heating or neutralization with antibody. The shut-off is faster and more effective in HSV-2-infected cells than in HSV-1-infected cells; this observation permitted the initial mapping of the genetic locus that confers upon HSV-1 × HSV-2 recombinants the accelerated shut-off characteristic of HSV-2 (122). More recently, isolation of *vhs* (virion host shut-off) mutants which fail to shut off host polypeptide synthesis in HSV-infected cells (395) has demonstrated more conclusively that this function is due to a virion protein (252,395).

The second stage, documented by Honess and Roizman (184,185), Fenwick and Roizman (124), Nishioka and Silverstein (334,335), and Silverstein and Engelhardt (468), requires *de novo* synthesis of proteins after infection. The shut-off coincides with the onset of synthesis of β proteins, but the experimental results do not exclude the possibility that the shut-off is caused by γ, rather than β, gene products.

Viral Genes Affecting Host Shut-Off

Structure and Expression of the vhs *Gene*

A rapid shut-off function was initially mapped to 0.52–0.59 (122). Isolation of a mutant defective in *vhs* function (395) allowed further mapping of the gene responsible. Mapping studies (253) have identified sequences from 0.604 to 0.606 on the viral genome as being responsible for the *vhs*$^-$ phenotype of the mutants. The open-reading frame in this region has been designated UL41, and it encodes a protein with an apparent molecular weight of 58,000 and a predicted molecular weight of 54,914 (294). A single mRNA has been shown to cross the minimal region shown to contain the mutations, 1.6 Kb in length with no introns (139). The RNA is expressed as a γ_1 gene.

Function of the vhs *Gene Product*

Early studies showed that virion components were responsible for destabilization and degradation of host mRNA (125). Further studies have shown that the virion component required for both mRNA destabilization and degradation is the *vhs* gene product. Furthermore, the *vhs* gene product is also responsible for a nondiscriminatory destabilization and degradation of viral α, β, and γ mRNAs (123,252,253,344,443,491). In cells infected with the *vhs*$^-$ mutant, host protein synthesis is not shut off. α and β protein synthesis are somewhat prolonged compared to wild type. Both of these effects have been shown to be due to a stabilization of host and viral mRNAs; in cells infected by *vhs*$^-$ mutants, mRNAs are not degraded as rapidly as in cells infected by wild-type virus.

Frenkel and associates (252) suspected that this function confers at least two advantages on the virus. First, it removes preexisting host mRNA from the pool of translatable messages, allowing the viral mRNAs to take over the pool rapidly. Second, destabilization of viral mRNAs allows a rapid transition from one regulatory class to the next. In the absence of the *vhs*

function, α and β proteins are produced beyond the time spans normally seen; the positive transcriptional controls discussed in the rest of this chapter are not enough to ensure efficient α-to-β and β-to-γ transitions. Although the *vhs*$^-$ mutation is not lethal, wild-type virus does have a growth advantage in tissue culture, indicating that efficient separation of the regulatory classes is helpful to the virus (252,395).

ICP47

The predicted translated and apparent molecular weights of ICP47 are 9,792 and 12,000, respectively. The gene appears to be nonessential for growth in cell culture. The α47$^-$ mutant phenotype apparent to date is the conservation of a host protein capable of binding with a high degree of specificity to a viral RNA transcribed across ori$_s$. In HSV-1-infected cells, the disappearance of this RNA-binding function is specifically associated with the production of functional ICP47 early in infection (428).

VIRULENCE

In healthy nonimmunocompromised humans, encephalitis occurs rarely (531). In experimental animals, it is frequently the only manifestation of disease. In studies on the molecular basis of disease induced by HSV, the endpoint of the research objective—the disease—is often synonymous with the destruction of the central nervous system (CNS). However, *neurovirulence*, as measured by intracerebral inoculation of virus, is clearly a misnomer. Wild-type HSV strains invariably multiply when injected into the CNS of experimental animals. Direct injection of virus into the CNS measures the capacity of the virus to grow and destroy an amount of CNS tissue that will result in death before the immune system blocks further virus spread. Because, in most instances, destruction of the CNS and death are related to virus multiplication (in quantitative terms), the CNS tissue specific growth, or *neurogrowth*, is measured in terms of the amount of virus required to reach a specific endpoint of tissue destruction.

A more rigorous attribute of virulence is invasiveness—the capacity to reach a target organ from the portal of entry. To disseminate to the target organ, it may be necessary for the virus to multiply at peripheral sites. In experimental systems, neurovirulence, the model of the disease producing the phenotype of HSV, is the consequence of (i) peripheral multiplication, (ii) invasion of the CNS, and (iii) growth in the CNS. Peripheral growth and invasiveness into the CNS can be quantified by measuring the amount of virus recovered (a) at the peripheral site and (b) in the CNS as a function of inoculum delivered to a peripheral site (i.e., footpad, eye, ear, etc.). The components of neurovirulence are not readily differentiable. *Neuroinvasiveness* can be differentiated from neurogrowth only in cases where the virus is capable of multiplying in the CNS (e.g., low PFU/LD$_{50}$ ratios after intracerebral inoculation of mice) and at peripheral sites but incapable of invading the CNS. Failure of the virus to grow in the CNS abolishes its virulence, but the loss of tissue-specific growth is quite distinct from the capacity to invade the CNS.

Wild-type isolates differ with respect to neurogrowth and neuroinvasiveness: In our experience, isolates from the brains of encephalitis patients require the lowest pfu/LD$_{50}$ ratios, as assayed by intracerebral inoculation in mice, whereas careful studies by Whitley and collaborators failed to differentiate between peripheral and CNS isolates from the same patients (R. J. Whitley, *personal communication*). Strains with elevated pfu/LD$_{50}$ ratios yield mutants with increased neurogrowth, and these can be readily selected by serial passage in the mouse brain. Mutants with increased neuroinvasiveness can also be selected by serial passage of virus isolated from the brain but inoculated at a peripheral site. It is our impression that this heightened neuroinvasiveness is, to some extent, inoculation-route-specific.

Cell culture correlates of neurovirulence do not exist. No differences are readily apparent in cell culture among wild-type viruses differing with respect to neurogrowth by a factor of 100 (i.e., between approximately 1 and 100 pfu/LD$_{50}$). Some mutants requiring 10^6 pfu/LD$_{50}$ may have a more restricted host range, reduced yield, or sluggish or multiplicity-dependent growth in cell culture (312,526).

In the past decade, virulence loci comprising both neurogrowth and neuroinvasiveness, or only one of these, have been ascribed to several sites, but particularly in or around the domain of the *tk* gene (126,312,381,481) and at the right terminus of the unique sequences of the L component in the P arrangement of HSV DNA (52,199,201,235,429, 502,503). At the *tk* locus, the neurovirulence may also be associated with genes other than *tk*, inasmuch as restoration of the *tk* gene in a *UL24*$^-$ mutant at another site did not result in increased capacity to cause death (B. Meignier and B. Roizman, *unpublished studies*). The major problem faced by most of these reports is as follows: Nearly any mutation or deletion introduced spontaneously or by design into the HSV genome results in reduced neurogrowth, and nearly any mutation or deletion introduced by default results in decreased virulence. Deletions and base substitutions in some genes have a more profound effect than those in other genes. For example, deletion of nearly any gene in the S component resulted in reduced capacity to grow in the CNS (312,526), and mutations in the ribonucleotide reductase subunit genes resulted in the same reduced capacity (42). It is obvious that any mutations (includ-

ing those that occur spontaneously and accumulate in laboratory strains characterized by a long history of serial passages in cell culture) and deletions that reduce the capacity to grow peripherally or in the CNS, or that reduce the capacity to invade the CNS, could be designated as "virulence loci," since rescue of the mutation or deletion will restore the phenotype of the parent virus [see, for example, the deletion in HSV-1 strain HFEM (235,429)]. Obviously, the genes that have been deleted or mutated are required for tissue-specific growth; their function is required, but is not sufficient, for neurovirulence in the context of the definition given here.

Also in the context of the definition of neurovirulence, none of the HSV genes tested to date, with the possible exceptions of gC and gE (52,207,312), appear to be dispensible with respect to replication in the CNS; this may also be true for other tissues. Genes specific for neuroinvasion have been reported, but these studies do not fully meet the stringent criteria for differentiation of neuroinvasiveness from failure to multiply efficiently.

It should be stressed that virulence is a multifactorial phenomenon reflecting the capacity of the virus to produce high yields and to spread in direct competition with the immune response whose objective is to block virus multiplication. Mutations to increased neurogrowth very likely enable (a) higher expression of viral genes whose functions are not complemented in the CNS and (b) more efficient molecular interactions of the viral genome or its gene products with the cellular factors required for viral replication. A central question is, Why do mutations for accelerated growth or for greater neuroinvasiveness not arise, since such mutants can be readily produced in the laboratory by serial passage?

Little is known of the mechanism by which HSV invades the CNS of human adults, whether by infection of a cranial nerve or by postsynaptic transmission. In principle, the epidemiologically significant virus in human-to-human transmission is that which appears on the mucous membranes or skin after first infection or after reactivation of latent virus. Although we have alluded to the observation that isolates from CNS tend to be virulent, selection for increased neuroinvasiveness is not a useful property inasmuch as the virus that multiplies in the CNS is less likely to be transmitted from person to person than by virus multiplying in the mucous membranes of otherwise healthy people.

LATENCY

The ability of HSV to remain latent in the human host for its lifetime is the unique and intellectually most challenging aspect of its biology. The virus enters sensory nerves innervating the cells infected at the portal of entry. In latently infected neurons, the viral genome acquires the characteristics of endless or circular DNA (133,315,404,405), may be in nucleosomal form (100), and has been reported not to be extensively methylated (103). To our knowledge, no functions are expressed which are required for the establishment of the latent state. In a fraction of those harboring HSV in a latent state, the virus is periodically reactivated; infectious virus is carried by axonal transport (70), usually to cells innervated by the infected neurons at or near the portal of entry (49,79,156,409). Depending on the host immune response, the resulting lesion may vary considerably in severity, from barely visible vesicles to rather severe, debilitating lesions in immunosuppressed individuals. The clinical aspects of latent infection and reactivation are discussed in Chapter 66. This section concerns the molecular biology of latency.

HSV Latency in Experimental Systems

The Experimental System

The most useful model systems are mice, guinea pigs, and rabbits. In the mouse, latent infection is readily established after eye, footpad, or ear inoculation, but the latent virus does not reactivate spontaneously (29,30,173,174,484). Latent virus in the rabbit does reactivate spontaneously (331). The guinea pig shows recurrent lesions after vaginal infection with high doses of HSV-2, but it is not clear whether these are the consequence of a festering, chronic infection or of a reactivated, truly latent virus (480). At the other extreme are latency models in cells cultured *in vitro*. The latently infected neuron is nonpermissive at the time it harbors the virus in the latent state. In the ganglion, the permissivity of the infected neuron is transient. When placed in culture, neurons become permissive. Those that contain latent virus activate its multiplication. It has been reported that neurons retain virus in a latent state in the presence of neuronal growth factor, and, conversely, the virus is activated when the growth factor is withdrawn (538). The issue is whether virus activation coincides with incipient neuronal death and whether permissivity for virus growth and maintenance of physiologic integrity are mutually exclusive.

A number of laboratories have reported the maintenance of the viral genome by rendering cells nonpermissive by a variety of methods (65,66,430,533–537). To our jaundiced eye, the state of nonpermissivity induced in cells in culture by elevated temperatures, interferon, or antiviral drugs is not equivalent to the nonpermissive state of the neuronal cells *in vivo*.

The events transpiring in animal models can be di-

vided into several stages. In the initial stage, virus replication ensues in the tissues at or near the site of inoculation. This initial multiplication ensures contact with, and entry into, the sensory nerve endings. The capsid is transported by rapid retrograde axonal flow to the neuronal nucleus (244,276). Data obtained from infection of neurons cultured *in vitro* indicate that the viral capsids are transported to neuronal nuclei by retrograde axonal transport involving microtubules. Drugs which disrupt neuronal microtubule structures, or which are known to inhibit retrograde transport of certain compounds, also inhibit the ability of the virus to move from the peripheral endings of neurons to the nuclei (244). Electron-microscopic studies indicate that in neurons infected in cell culture, the viral particle that is being transported is the unenveloped capsid (276).

We suspect that the initial multiplication is (a) not essential if the virus comes in contact with nerve endings and (b) critical if the virus is merely deposited on the surface of the peripheral tissues.

In some animal models, there is a short period of viral replication in the ganglia at this stage (226–230,301,302,382,383,508,509,520,542); however, this may be an artifact of the large amount of virus used in the inoculum to attain a high percentage of latently infected ganglia.

In the second stage, at a maximum of 2–4 weeks after inoculation, no replicating virus can be detected in the sensory ganglia innervating the site of inoculation.

In the last stage, certain stimuli (e.g., physical or emotional stress, peripheral tissue damage or intake of certain hormones in humans, and both peripheral tissue damage and administration of drugs that stimulate prostaglandin synthesis in experimental animals) may result in activation of virus multiplication concurrent with axonal transport of the virus progeny, usually to a site at or near the portal of entry. Although the issue is still being debated frequently and hotly, there is little doubt that virus multiplication results in destruction of the neuronal cell.

Viral Gene Expression in Latently Infected Neurons

Extensive studies on ganglia harboring latent HSV have been rewarded by an extreme paucity of evidence for viral gene expression. The only transcript detected to date is one designated optimistically as latency-associated transcript 1 (LAT1) (486). This transcript is abundant and accumulates in the nuclei of neurons of latently infected animals and humans (243,406,483, 485,486). LAT1 is spliced (517,527), and in latently infected cells it is not polyadenylated (516). It has been reported that the LAT1 population is heterogeneous, varying in the donor/acceptor splicing sites, and hence capable of expressing more than one protein—provided, of course, that it were transported to the cytoplasm (517,527). Because it is, in part, complementary to the 3′ terminus of the α0 mRNA, it has been thought that the function of LAT1 is to preclude the expression of α0 (486). Confounding the issue is the observation that *LAT1*$^-$ mutants are capable of establishing latency (200).

In a different category is the observation that in trigeminal ganglia harboring latent virus, there are between 0.1 and 1 viral genome equivalents per cell genome (40,384,404,405). This datum poses an intriguing question. Heretofore, the number of neurons harboring virus was thought to be between 0.1% and 3% of total neurons. Even assuming a 10% total, the number of neurons harboring virus would constitute less that 1% of all ganglionic cells. To account for the high number of viral genomes per cell harboring latent virus, it is necessary to postulate the following: (i) More than one viral genome can enter and establish latency in the same neuron, or (ii) viral genomes are amplified by the cellular machinery during the latent state (422).

The Role of Viral Multiplication in the Establishment and Assessment of the Latent State

There are several important facets of latent infections which relate to the role of virus multiplication, both at the periphery and in the neurons harboring the virus.

1. As noted above, HSV must have access to the nerve endings in order to establish latency, and therefore it could be expected that the greater the number of peripheral cells that become infected and support virus multiplication, the larger the number of neurons which will harbor latent virus. The relevant phenomenon in humans is that the frequency of reactivations resulting in recrudescences of lesions is related to the severity of lesions caused by the first infection. In the model we have proposed (422) and have elaborated below, the frequency of recurrences would be determined, in part, by the number of neurons harboring virus.

2. Several years ago, it was proposed that the latent virus makes a "round trip"; that is, the reactivated virus reestablishes the latent state by infecting the nerve endings of hitherto uninfected neurons (225). This hypothesis is not tenable. First, in experimental systems, it is very difficult to superinfect ganglia harboring latent virus with a second, marked virus (51,314). Perhaps even more significant, the "round trip" does not appear to take place in humans even under conditions that would favor such a phenomenon. Thus, in a small number of individuals, mutants that

were both virulent and acyclovir-resistant have arisen (110,349). Recurrent lesions that emerged after the mutant was eliminated with the aid of other drugs did not contain the acyclovir-resistant virus. While this phenomenon has been attributed to rapid elimination of the peripheral infected cells by the immune system (51), the observations may have more profound implications inasmuch as induction of latent virus should eliminate it from the ganglion.

3. The operational definition of latent virus is useful but self-limiting, in a rather significant fashion. Currently, latent virus is defined as that which is detected after incubation of intact ganglionic tissue with suitable susceptible cells and not by inoculation of the susceptible cells with macerated ganglia. While adherence to this operational definition is critical, in experimental animals and in humans, viral DNA can be detected in CNS tissue (particularly in brain stem), but infectious virus cannot be reactivated from these tissues. It has been customary to ignore these genomes or assume that the brain stem accumulates defective genomes. The possibility that this is not the case, that the neuronal population of the brain stem represents a population that is nonpermissive, must be considered in light of two observations. Foremost, reactivations are more readily demonstrable with so-called "virulent" (or, by the definition above, capable of heightened neurogrowth) strains than with relatively avirulent strains. Of greater potential significance is the recent observation that certain deletion mutants are not reactivated. While failure of $\alpha 0^-$ mutants to reactivate from neurons of ganglia known to contain viral DNA has been attributed to a key role of the $\alpha 0$ gene in reactivation (264), similar results have been seen with tk^- viruses (see below). Competence to reactivate may reflect a myriad of functions that include both those that are specific to the termination of the latent state and those related to the overall capacity of the virus to multiply in an extended host range—the relatively nonpermissive sensory neuron. These two sets of functions may be difficult to differentiate.

Establishment and Maintenance of the Latent State: The Data

Viral Gene Expression Required for the Establishment of Latency

The viral genome during latency has been reported to be in an "endless" form, either concatemeric or circular (i.e., in a state similar to that seen immediately after infection) (404,405). In cells in culture, acquisition of the circular or concatemeric form immediately after infection does not require *de novo* protein synthesis (362), and therefore the presence of circular DNA does not imply viral gene expression.

Reactivation of virus, as noted above, is not a reliable indicator of the ability to establish latency. If we accept the operational definition that the presence of LAT1 RNA is indicative of the latent state, it follows that any virus capable of infecting neurons is able to establish latency. To date, all HSV mutants (except those with deletions in the LAT1 sequence) shown to be capable of peripheral replication also appear to induce LAT1 in neurons of ganglia which innervate the site of inoculation (e.g., 63,312,313). Since mutants in all HSV genes have not been tested, the conclusion that no viral gene function is specifically required for the establishment of latency may be prognostic but premature.

Viral Gene Expression Required for the Maintenance of Latency and for the Activation of Viral Multiplication

Studies on the viral gene expression required for maintenance of the latent state or for activation of viral multiplication suffer from a peculiar operational problem: Inactivation of a gene essential for either process should result in failure to reactivate. Failure to reactivate virus from ganglia of experimental animals which had been inoculated with adequate amounts of virus by an appropriate route could be due to (i) failure to establish latency, (ii) failure to maintain the latent state, or (iii) failure of the latent genomes to be induced. Detection of viral genomes or of LAT1 in neurons eliminates, but does not discriminate between, alternatives (i) and (ii), but it introduces another alternative—namely, that the viral DNA retained in the ganglia and expressing LAT1 represents defective genomes. While appropriate experimental designs can surmount this problem, this has not been done so far.

Viruses which are capable of independent replication and which failed to be reactivated readily in mice are tk^- and $\alpha 0^-$ mutants. The ability of tk^- virus to establish latency has been disputed. While Tenser and colleagues (500,501) have reported that tk^- viruses cannot establish latency in mice and have ascribed a significance to that finding, other workers have reactivated viruses with little or no TK activity from mice, and even deletion mutants have been reactivated from latently infected rabbits (50,157–159,312,451). $\alpha 0^-$ mutants may fall into a similar category. Although they are not readily inducible in mice (264), viral DNA was detected in ganglia (264), and spontaneously reactivated virus was isolated from a rabbit (Y. J. Gordon, *personal communication*). The LAT1 RNA can be detected in neurons of mice infected with $\alpha 0^-$ (D. M. Knipe, *personal communication*) or tk^- (63) viruses.

As noted earlier in the text, the ability to reactivate may well reflect the ability of the virus to grow in re-

strictive cells. In experimental animal systems, there is the added requirement that viruses multiply effectively and rapidly, since viral multiplication and dissemination of the reactivated virus is in competition with the immune systems whose object is to ablate it. Examination of the results of reactivation from trigeminal ganglia of deletion mutants inoculated into mice is consistent with the hypothesis that mutations that affect growth of virus in cells also affect the ability of the mutants to be reactivated to a level high enough to be detected.

Reactivation of Virus in Experimental Systems

In humans, latent virus is reactivated after (a) local stimuli such as injury to tissues innervated by neurons harboring latent virus or (b) systemic stimuli such as physical or emotional stress, menstruation, hormonal intake, and so on, which may reactivate virus simultaneously in neurons of diverse ganglia (e.g., trigeminal and sacral). In experimental systems, induction of latent virus multiplication has been induced by (a) physical trauma to tissues innervated by the neurons harboring virus (8,165), and (b) iontophoresis of epinephrine (250,251) or other drugs (157,166,463). The molecular basis of reactivation, as well as the order in which viral genes are induced, is not known.

Establishment and Maintenance of the Latent State: A Model

The molecular basis of latency rests on answers to several key questions: (i) Since HSV readily multiplies in a variety of cells derived from human or animal tissues, why does lytic infection not ensue in neurons harboring latent virus? (ii) At what stage in the reproductive cycle is viral multiplication arrested? (iii) What is the origin of the rather high number of copies of the viral genome per latently infected neuronal cell? (iv) Why are all neurons not reactivated at the same time? (v) At what stage in the cascade of viral gene expression does replication of latent virus begin? (vi) Why is HSV-2 more readily reactivated in sacral ganglia, whereas HSV-1 is more readily reactivated from trigeminal or cervical ganglia? Our model is based primarily on the hypothesis that latency is required for the perpetuation of the virus in its natural host population and that the virus has evolved elaborate mechanisms to control the latent state.

The model we propose, largely for its heuristic value, is an extension of the one we described earlier (422) and consists of several components:

1. The model proposes that two distinct mechanisms operate to block the expression of HSV in neuronal cells. Thus, previously published data indicate that αTIF and capsids reach the nucleus independently inasmuch as viral mutants which do not release DNA at the nuclear pore do induce a reporter gene linked to an α promoter (21). The separation of the viral DNA from the *trans*-acting factor that induces the α genes is difficult to explain except in the context that it might be desirable for the virus not to multiply under certain conditions. Unlike the epithelial cell at the portal of entry with a distance of 10 μm or less between the plasma and nuclear membranes, in the infected sensory cells the distance between nerve endings and the nuclear membrane may well be measured in centimeters. In the absence of the αTIF, gene expression may not occur, or it would be grossly retarded. The absence of αTIF may not be sufficient to enable the the infected neurons to block viral expression. A second mechanism would be predicted to block the expression of α genes.

2. The model also proposes that activation of virus multiplication is the consequence of the cumulative effect of stimuli to which each cell harboring virus responds independently. Specifically, the hypothesis envisions that both local and systemic stimuli cause the viral DNA copy number to be increased. Virus would become activated when the copy number exceeds a certain threshold. Since the effect of the stimulus, the increase in the DNA copy number, and the precise threshold may vary from cell to cell, not all cells would be activated simultaneously.

3. The increase in the DNA copy number may not, per se, ablate the block in virus multiplication. The added requirement for viral multiplication falls under the heading of capacity for gene expression and is poorly defined. A common feature of this property is seen in the case of deletion mutants (e.g., $\alpha 22^-$; see ref. 450) infecting nonpermissive or restrictive cells. Such mutants often exhibit a multiplicity dependence reflected in the failure of virus multiplication at low, but not high, multiplicities of infection. The additional functions that may be required to achieve clinically detectable reactivation (or detectable amounts of infectious virus in experimental systems) may be those of a set of genes. The model proposes the following: (a) This set would include genes that are required, as well as those that are dispensable, for multiplication in cells in culture, and (b) the expression rate and product abundance of these genes would determine whether infection is productive or abortive in the particular cell in which the virus is latent.

HSV-1 and HSV-2 are very closely related viruses which have predilections for oral and genital mucosa, respectively. It could be argued that the differences in the nucleotide sequences of their genomes and in the amino acid sequences of their proteins reflect the differences in the environments in which they function. Conversely, the differences in the reactivation rates of HSV-1 and HSV-2 in trigeminal and sacral ganglia

must reflect the differences in the respective ganglia. The differences may be reflected not in the ability to establish latency but in the gene expression required to overcome the block to effective viral gene expression.

CONCLUSIONS

The studies on herpes simplex viruses are, at last, entering a most exciting stage, largely because the words "structure and function" are beginning to have an operational meaning. As a field of endeavor, we are beginning to characterize the interaction of proteins among themselves and with viral nucleic acids. In addition, the host factors crucial to virus multiplication, and potentially to latency, are being sought out. The armamentarium for a major assault on the mysteries underlying the biology of these viruses is in place, reflecting the contributions of many laboratories over many years.

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ANIMAL VIRUS DNA REPLICATION[1]

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CONTENTS

PERSPECTIVES AND SUMMARY

Much of the impetus for studying the replication of animal virus genomes comes from a desire to understand the events that occur during the replication of eukaryotic chromosomes. Viruses offer many advantages for the study of

eukaryotic DNA replication. Viral genomes are relatively simple and can be readily manipulated by modern genetic methods. In addition, the replication of some viral genomes has proven amenable to analysis in cell-free systems. These facts significantly enhance the ability to analyze replication mechanisms at the molecular level. There are a number of potentially useful viral systems, and this review focuses on four of the best characterized: (*a*) adenovirus, (*b*) SV40, (*c*) herpes simplex virus, and (*d*) bovine papillomavirus. Each system has certain unique virtues that can be exploited to gain insight into different aspects of the replication process.

Adenovirus DNA replication occurs by a process that is significantly less complex than chromosomal DNA replication. Replication initiates by a novel protein priming mechanism, and all daughter strands are elongated by a continuous mode of synthesis such as occurs at the leading strand of a chromosomal replication fork. The biochemical dissection of a soluble in vitro system capable of faithfully replicating adenovirus DNA has led to the identification of most of the proteins involved. Adenovirus DNA replication requires the participation both of virus-encoded replication proteins and host-cell–encoded transcription factors. Since a linkage between replication and transcription has now been observed in other systems, it is likely that further analysis of the adenovirus system will provide insights that are of general importance.

The SV40 genome represents a more complete model system for studying cellular DNA replication. SV40 encodes only a single replication protein (T antigen) and relies predominantly on the host-cell replication machinery. In vivo studies have established that many of the details of SV40 DNA replication are closely similar to those of cellular DNA replication. Replication initiates at a fixed site on the viral genome and proceeds bidirectionally with continuous growth of leading strands and discontinuous growth of lagging strands. As in the case of adenovirus, an efficient cell-free replication system has been developed for SV40, and dissection of this system has identified several cellular replication proteins. A partial understanding of the mechanisms by which these proteins act is beginning to emerge, and it seems certain that this will be an area of continued rapid progress.

In contrast to SV40, herpes simplex virus (HSV) encodes many, if not all, of the proteins that are involved in the replication of its genome. Thus, HSV DNA replication has been studied by using a combination of genetics and biochemistry. The complete set of viral genes necessary for DNA synthesis has recently been identified, and the products of many of these genes have been purified and partially characterized. Several of these purified proteins have functions expected of replication proteins, including a DNA polymerase, a helicase, a primase, a single-stranded DNA binding protein, and an origin recognition protein. It seems likely that the availability of these purified

proteins will soon lead to the development of an in vitro system capable of specifically replicating HSV DNA. Genetic and biochemical dissection of such a system should provide important new insights into the molecular mechanisms of eukaryotic DNA replication.

Adenovirus, SV40, and HSV are all examples of viruses that normally multiply by productive cytocidal infection. In all of these cases, viral DNA replication begins soon after infection and continues at a high rate until the death of the host cell. In contrast, bovine papillomavirus (BPV) represents an example of a virus that is capable of multiplying as a stable extrachromosomal element. In this case, viral DNA replication is controlled so that the number of viral genomes doubles only once per cell cycle, and under normal circumstances the host is not killed. As in the case of SV40, the BPV genome is relatively small and encodes only a small number of proteins involved in DNA replication; viral DNA synthesis depends heavily on host-cell replication proteins. The biochemical analysis of BPV DNA replication is in its infancy, but genetic analyses have provided evidence for a negative control system that apparently ensures that each viral genome is replicated once and only once during each cell cycle. Bovine papillomavirus therefore represents an excellent model for illuminating mechanisms involved in regulating DNA replication.

ADENOVIRUS DNA REPLICATION

Adenovirus DNA replication is better understood than the replication of the other animal virus genomes because it was the first to be established in vitro (1). Although many of the molecular details remain to be worked out, the basic features of the adenovirus DNA replication pathway are now reasonably clear, and most of the proteins involved in the replication process have been identified (2–5). On the basis of the information gathered to date, it is evident that the adenoviruses have evolved some very interesting and novel solutions to the replication problem, including the use of a "protein priming" mechanism for initiation and the diversion of cellular transcription factors to the viral replication machinery. It seems certain that further biochemical analysis of this system will illuminate and extend our understanding of DNA replication in the context of the animal cell.

The Adenovirus Chromosome

The genomes of the human adenoviruses are double-stranded linear DNA molecules containing approximately 35,000 base pairs. The 5' terminus of each strand of the viral genome is covalently attached to a virus-encoded protein (TP) with a molecular weight of about 55,000 (6, 7). In addition, the nucleotide sequences at the extreme ends of the genome are identical (8, 9).

Both of these structural features play important roles in the initiation of viral DNA replication.

Numerous in vivo studies have established that adenovirus DNA replication takes place in two stages (2–5). In the first stage, DNA synthesis is initiated at either terminus of the duplex viral genome by the protein priming mechanism (see below). The initiation process results in the establishment of a replication fork that moves from one end of the genome to the other. At each replication fork only one of the two parental DNA strands serves as a template for DNA synthesis. Thus, the products of the first stage of replication are a daughter duplex and a displaced single strand. In the second stage of DNA replication, the strand complementary to the displaced single strand is synthesized. It seems likely that the initial step in this process is the circularization of the single-stranded template by annealing of its self-complementary termini. The resulting duplex "panhandle" has the same structure as the terminus of the duplex adenovirus genome and is presumably recognized by the same initiation machinery that operates in the first stage of replication. Following a second initiation event, complementary strand synthesis proceeds from one end of the template to the other, generating a second daughter duplex. In both stages of adenovirus DNA replication there is only one priming event per nascent daughter strand, so all viral strands are synthesized in a continuous fashion from their 5' termini to their 3' termini.

The adenoviruses encode three proteins that play central roles in viral DNA replication: the terminal protein precursor (pTP), the adenovirus DNA polymerase (Ad pol), and the single-stranded DNA binding protein (DBP) (10–12). Together these three proteins consume approximately 25% of the coding capacity of the viral genome. The mRNAs for all three proteins are products of the same viral transcription unit and are produced by differential splicing of a common precursor (13). This genetic organization provides a simple mechanism for the coordinate regulation of the levels of replication proteins during infection. In addition to the virus-encoded proteins, adenovirus DNA replication requires the participation of several cellular proteins. Those identified to date include two cellular transcription factors (NF-I/CTF and NF-III/OTF-1) and a cellular topoisomerase activity (14–20). The biochemical roles of these viral and cellular replication proteins are discussed below.

Initiation of Adenovirus DNA Replication

THE PROTEIN PRIMING MODEL Initiation of adenovirus DNA replication occurs by a novel mechanism in which the first nucleotide in the new DNA chain becomes covalently linked to a virus-encoded protein, the terminal protein precursor. This mechanism is unique among the DNA viruses of mammals, but a similar mechanism operates during the replication of other

chromosomes, for example bacteriophage $\Phi 29$ (21). The protein priming model was first proposed following the discovery that the 5' ends of adenovirus DNA strands are covalently linked to the 55-kd terminal protein (22). Direct biochemical support for the model was obtained by analysis of the initiation reaction in the adenovirus cell-free replication system. Initial studies demonstrated that the adenovirus terminal protein is synthesized in the form of a larger 80-kd precursor (pTP), which is active in initiation (10, 13, 23, 24). The pTP is processed by proteolysis to the mature 55-kd form during packaging of the viral genome into virions (23). A series of isotope transfer experiments provided evidence that the critical first step in the replication reaction is the formation of an ester bond between the β-OH of a serine residue in the pTP and the α-phosphoryl group of dCMP, the first residue in the new DNA chain (10). The nascent strand then grows by extension from the 3' hydroxyl of the covalently bound dCMP residue (22). The subsequent development of a direct assay for the formation of a covalent complex between dCMP and the pTP (pTP-dCMP) made it possible to purify the pTP in functional form and to define the requirements for the initiation reaction (25–28). Work in a number of laboratories has shown that initiation is dependent upon the presence of specific nucleotide sequence elements at the termini of the viral genome and requires the participation of several viral and cellular proteins.

THE ADENOVIRUS ORIGIN OF DNA REPLICATION The natural template for adenovirus DNA replication in vivo or in vitro is the viral chromosome with the covalently attached terminal protein (TP or pTP). However, as first demonstrated by Tamanoi & Stillman (28), plasmids containing the cloned adenovirus terminal sequence will support initiation of DNA replication in vitro, provided that the plasmid is cleaved with a restriction enzyme in such a way that the adenovirus terminus is located near the end of the resulting linear DNA molecule. This observation provided definitive evidence that specific nucleotide sequence elements in the viral genome are recognized by the initiation machinery. The efficiency of initiation with plasmid templates is considerably lower than that observed with adenovirus chromosomes isolated from purified virions. Moreover, recent studies have revealed that the protein and cofactor requirements for initiation are somewhat different for the two templates (29–32). However, analysis of plasmids with deletion and/or base substitution mutations has been very useful for defining the nucleotide sequence requirements for initiation.

Most in vitro studies of the nucleotide sequence requirements for adenovirus DNA replication have been conducted with serotypes 2 or 5. Analysis of a large number of deletion and base substitution mutations has revealed that the adenovirus origin of DNA replication is complex, containing at least three

functionally distinct domains (17, 33–42). Domain A consists of the first 18 base pairs of the viral genome and represents the minimal origin of replication. The presence of domain A is absolutely required for initiation of adenovirus DNA replication, but templates containing only domain A initiate DNA synthesis at a very low efficiency. All adenovirus serotypes that have been examined share a common 10-base-pair sequence, ATAATATACC, within this region of the viral genome (34, 43, 44). It has been suggested that this conserved motif is important for the binding of the virus-encoded initiation proteins to the origin (32, 45). Domains B and C, while not absolutely required for initiation of adenovirus DNA replication, contribute significantly to the efficiency of the initiation reaction. Domain B consists of the segment between nucleotides 19 and 39 (36, 37, 40, 42). As in the case of domain A, there is considerable sequence conservation in this region among the various adenovirus serotypes. Many (but not all) adenovirus genomes contain a version of the consensus sequence TGG(A/C)NNNNNGCCAA. As described below, this motif is recognized by a cellular DNA-binding protein, nuclear factor I(CTF)(14–16). The presence of domain B increases the efficiency of initiation of adenovirus 5 DNA replication at least 10-fold. Domain C of the adenovirus origin includes nucleotides 40 to 51 and contributes an additional factor of three to the efficiency of initiation of viral DNA replication in vitro (17, 42). The consensus sequence, AT(G/T)N(A/T)AAT, has been identified in this region. A second cellular DNA-binding protein, nuclear factor III (ORP-C, OTF-1), recognizes this sequence (17–19). The spacing between the minimal origin and domain B appears to be critical for origin function (41, 42). The insertion or deletion of only a few base pairs between the two segments dramatically reduces the efficiency of initiation. This observation suggests that the initiation reaction may require relatively short-range interactions between the protein factors that bind to the various domains of the origin.

Analysis of the replication of deletion mutants in vivo is largely consistent with the general picture of the sequence organization of the adenovirus genome derived from the in vitro studies (46–51). Both the conserved sequence element in domain A and the nuclear factor I binding site in domain B have been shown to be essential for adenovirus 2 (or 5) DNA replication in cultured cells. The stimulatory effect of domain C has not yet been observed in vivo. In contrast to most adenovirus serotypes, the replication origin of adenovirus type 4 lacks a recognition site for nuclear factor I. It has been demonstrated that Ad4 DNA replication, both in vivo and in vitro, requires only the terminal 18 base pairs of the genome, which are identical to the minimal origin of Ad2 or Ad 5 (49, 52).

CELLULAR ORIGIN-BINDING PROTEINS Nuclear factor I (NF-I) was originally identified as a cellular factor that stimulated formation of pTP-dCMP

complexes by partially purified viral replication proteins (14). The stimulatory activity was subsequently purified to near homogeneity by recognition site affinity chromatography and shown to consist of a family of polypeptides with molecular weights between 52,000 and 66,000 (15). Recently, three human cDNA clones of NF-I have been isolated and characterized (53). The clones contain blocks of identical sequence interspersed with blocks of different sequence, suggesting that the corresponding mRNAs are generated by differential splicing. Analysis of the open reading frames in the clones suggests that each mRNA encodes a distinct protein. Thus, differential splicing may account, at least in part, for the multiplicity of NF-I polypeptides that have been observed. Although it has been demonstrated that the protein products of all three NF-I cDNA clones are active in stimulating adenovirus DNA replication in vitro, it is not yet clear whether all of the NF-I polypeptides are functionally equivalent (53).

The interaction between NF-I and its recognition sequence has been studied using chemical probes and in vitro mutagenesis (13, 39, 54). Taken together, these studies suggest that the optimal recognition site consists of the symmetrical sequence TTGGCN$_5$GCCAA. The principal contacts between protein and DNA are in the major groove, and nearly all of the contacts are accessible from one side of the helix (54). Given the symmetry of the protein-DNA contacts, it seems likely that NF-I binds as a dimer.

Purified nuclear factor I stimulates initiation of adenovirus DNA replication in vitro at least 30-fold (15, 42). The binding of NF-I to its recognition sequence is essential for the stimulatory effect, since base substitution mutations in the viral origin that abolish binding greatly reduce the efficiency of initiation (18, 36, 37, 39, 40, 42). The precise role of NF-I in the initiation reaction is not yet clear (see below).

Nuclear factor III (ORP-C) was identified as a stimulatory factor that recognized the sequence element TATGATAAT within domain C of the adenovirus 2 origin of replication (17, 18). The factor has been purified to homogeneity by recognition site affinity chromatography and shown to consist of a 92-kd polypeptide (55). Experiments with various chemical probes indicate that the protein makes both major and minor groove contacts with the DNA and that the contacts are not confined to one side of the helix (56). The binding site for NF-III is very close to that for NF-I; both proteins contact the same A/T base pair at position 39 in the adenovirus origin of replication (56). Despite their proximity, there is no evidence for cooperativity in binding (18, 56). Both NF-I and NF-III are required for optimal levels of DNA replication in vitro (17, 18, 55, 56), but the requirement for NF-III in vivo has not yet been demonstrated.

Interestingly, both nuclear factor I and nuclear factor III also appear to function as cellular transcription factors. A number of viral and cellular promoters contain functionally significant sequence elements that are related

to the NF-I recognition sequence (57–67). A protein factor (CCAAT transcription factor or CTF) that recognizes the GCCAAT motif present in several such promoters has been purified to homogeneity and shown to be capable of stimulating transcription of the human α globin gene in vitro and in vivo (68). Purified CTF was found to consist of a series of polypeptides with molecular weights similar to those previously described for NF-I (15). Detailed comparison of the physical and biochemical properties of CTF with those of NF-I demonstrated that the two groups of proteins are indeed identical (16).

The recognition site for nuclear factor III is similar to the octamer sequence that has been implicated in the transcriptional regulation of several genes, including the histone H2b, immunoglobulin, and small nuclear RNA (U1 and U2) genes (17, 18). Binding studies have demonstrated that NF-III binds to the promoter/enhancer regions of several such genes (69). A 92-kd protein factor (octamer transcription factor or OTF-1) that recognizes the octamer sequence in the histone H2b promoter and markedly stimulates H2b transcription in vitro has been purified from HeLa cells (70). The purified OTF-1 protein is physically and biologically indistinguishable from NF-III (19). The implications of the finding that cellular sequence-specific DNA-binding proteins can participate in both transcription and DNA replication are not yet clear. On the one hand, adenovirus may have simply subverted cellular transcriptional factors for its own purposes. On the other hand, there is abundant circumstantial evidence for a fundamental relationship between transcription and replication in eukaryotic cells (5, 71–73). Indeed, several examples of transcriptional signals that significantly affect the efficiency of DNA replication are documented in other sections of this review. Further study of the roles of NF-I and NF-III in adenovirus DNA replication may lead to better understanding of the mechanistic role of transcriptional factors in DNA replication.

REQUIREMENTS FOR THE INITIATION REACTION Initiation of adenovirus DNA replication is assayed in vitro by measuring the formation of a covalent complex between dCMP and the 80-kd pTP (25–28). The initiation reaction is absolutely dependent upon the presence of a DNA template. The most efficient template is the adenovirus chromosome containing the covalently attached 55-kd terminal protein, although other DNA molecules, such as linear plasmids or single-stranded DNA molecules, will support pTP-dCMP complex formation to some extent (see below). With adenovirus chromosomes as template, optimal initiation requires a minimum of four proteins: two cellular proteins, NF-I and NF-III (14, 15, 17, 18), and two virus-encoded proteins, pTP and the 140-kd adenovirus DNA polymerase (11, 74–76). The viral proteins copurify through several chromatographic steps,

and their sedimentation behavior suggests that they exist in a 1:1 complex (11, 74, 75). The complex can be separated into the individual polypeptides by glycerol gradient sedimentation in the presence of urea (74, 75, 77). This has made it possible to demonstrate the absolute requirement for both the pTP and the DNA polymerase in the initiation reaction (74, 75, 77). Initiation also requires ATP, which appears to be serving an effector function, since nonhydrolyzable analogues also stimulate initiation, and no ATP hydrolysis has been detected (32). It has been reported that a third virus-encoded protein, the 72-kd single-stranded DNA-binding protein (DBP) (78), stimulates initiation several-fold, but the protein is clearly not an essential participant in the reaction (18, 32).

As described above, duplex templates that lack the covalently attached terminal protein (e.g. linear plasmids containing the adenovirus origin of DNA replication at one terminus) are capable of supporting the initiation reaction, albeit at lower efficiency than adenovirus chromosomes. With such templates, an additional protein, factor pL, is required for efficient formation of pTP-dCMP complexes (29, 30). Factor pL has been purified to near homogeneity and shown to be a 44-kd polypeptide with 5' → 3' exonuclease activity (30). The pL exonuclease appears to activate adenovirus templates that lack the terminal protein by degrading the 5' end of the DNA strand that is normally displaced during adenovirus DNA replication (30, 31). This creates a short single-stranded region at the 3' end of the DNA strand that normally serves as the template for adenovirus DNA synthesis. Similar partially single-stranded templates, constructed using synthetic oligonucleotides, support the initiation reaction in the absence of factor pL (31). Thus, the presence of a single-stranded region at the 3' end of the template strand appears to allow the system to bypass the requirement for the 55-kd terminal protein on the input DNA. One possible interpretation of this result is that the 55-kd terminal protein attached to adenovirus chromosomes plays some role in opening the duplex during the early stages of the initiation process (see below). Except for the requirement for factor pL, the protein requirements for initiation on partially single-stranded templates are the same as those for adenovirus chromosomes (32). However, ATP is no longer required (32).

Single-stranded DNA molecules will also support formation of pTP-dCMP complexes in vitro (28, 29, 33, 35, 79). The sequence requirements for initiation with single-stranded templates appear to be somewhat less stringent* than with adenovirus chromosomes or plasmid DNAs (35). A large number of single-stranded DNA molecules, including those lacking the specific adenovirus origin sequences, have been observed to support pTP-dCMP complex formation with varying degrees of efficiency. However, it has been reported that an oligonucleotide containing the template strand of the adenovirus origin is 5–20 times as active in initiation as other single-stranded DNA molecules

*more than somewhat - See Dobbs et al Virol 178 : 43 '90 - may have no relationship to in vivo situation

(32, 80). With single-stranded DNA templates, initiation is dependent on the virus-encoded replication proteins, but is not dependent on NF-I or NF-III (32). Both ATP and the adenovirus DBP are inhibitory.

MECHANISM OF INITIATION The precise order of events during initiation of adenovirus DNA replication is not yet clear. The finding that single-stranded or partially single-stranded templates can support pTP-dCMP complex formation suggests that initiation is a two-step process (Figure 1). In the first step, the terminal region of the viral genome is unwound, exposing a short single-stranded region. In the second step, a dCMP residue is covalently linked to the pTP. Elucidation of the protein requirements for localized unwinding at the adenovirus origin must await the development of a direct unwinding assay. However, the available data suggest that the 55-kd terminal protein attached to the template DNA may play a role in unwinding, since the requirement for the terminal protein is obviated by presence of a single-stranded region at the end of the template. It is also possible that NF-I, NF-III, pTP, or the adenovirus polymerase participate in strand opening within the origin. The binding of the pTP and adenovirus DNA polymerase to the template strand of the origin presumably takes place following (or perhaps during) the unwinding reaction. There is some evidence that the complex of pTP and Ad polymerase may interact with sequence elements in the origin, but these interactions must be of relatively low specificity. It is possible that one role of NF-I and NF-III is to facilitate the binding or positioning of the pTP and adenovirus DNA polymerase on the DNA template. The final step in initiation, the formation of the covalent pTP-dCMP complex, probably occurs once the pTP-Ad pol complex is correctly positioned on the exposed template strand. Although both the pTP and the adenovirus DNA polymerase are required for the initiation reaction, an important unresolved question is whether the adenovirus DNA polymerase catalyzes the transfer of dCMP to the pTP or whether this is an autocatalytic process.

Elongation of Nascent DNA Chains

The synthesis of full-length adenovirus DNA strands in vitro requires the pTP, the Ad DNA polymerase, the Ad DBP, and nuclear factors I–III. At present there is good evidence that three of these proteins (Ad DNA polymerase, Ad DBP, and nuclear factor II) are directly involved in chain elongation (20, 81, 82). Although there is no direct evidence for the involvement of the other adenovirus replication proteins in elongation, this possibility cannot be completely ruled out. The adenovirus DNA polymerase is a 140-kd protein with physical and biochemical properties distinct from the other known eukaryotic DNA polymerases (11, 74, 81, 82). The enzyme is capable of utilizing a variety of deoxyribonucleotide homopolymer template-primers,

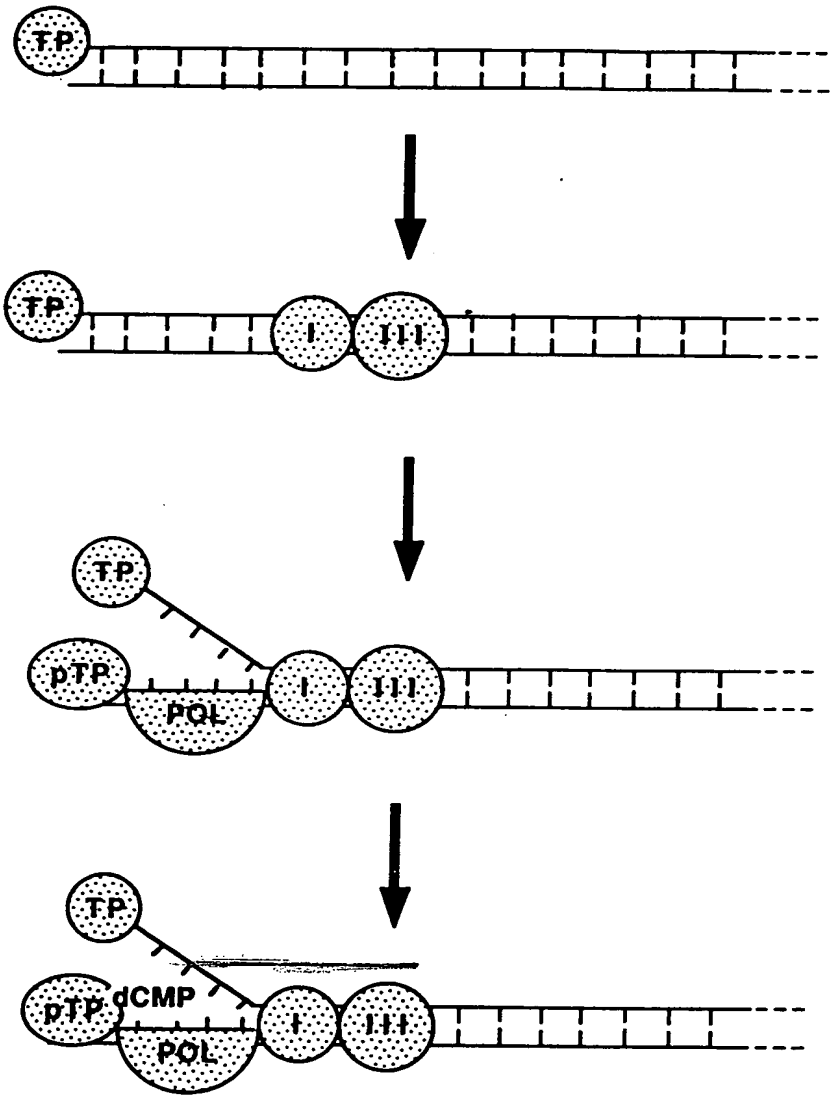

Figure 1 Diagrammatic representation of the initiation of adenovirus DNA replication. See text for details. Abbreviations used: I, nuclear factor I; III, nuclear factor III; TP, terminal protein; pTP, preterminal protein; POL, adenovirus DNA polymerase.

but is relatively inactive with RNA primers (82). Polymerase activity is inhibited by dideoxynucleotides and is resistant to aphidicolin. The purified polymerase contains an intrinsic 3'→5' exonuclease activity that is specific for single-stranded DNA and probably serves a proofreading function during polymerization (82). The Ad DBP is a 59-kd phosphoprotein that migrates in SDS-polyacrylamide gels with an apparent molecular weight of 72,000 (12, 83). The DBP binds tightly and cooperatively to single-stranded DNA in

sequence-independent fashion (12, 83–87). At saturation approximately one molecule of DBP is bound for every seven nucleotides (85). With poly(dT) as template and oligo(dA) as primer the DBP stimulates DNA synthesis by the Ad DNA polymerase as much as 100-fold (82). The stimulatory effect is quite specific, since the *Escherichia coli* SSB has no effect on Ad DNA polymerase activity and the Ad DBP does not stimulate the activity of other DNA polymerases such as HeLa DNA polymerase α. Based upon these and other results (81, 82), it seems likely that there is a highly specific interaction between the DBP and the Ad DNA polymerase that increases the efficiency of polymerization; however, a stable complex of the two proteins has not yet been detected. In the presence of the DBP, the Ad polymerase is a highly processive enzyme, capable of synthesizing DNA chains at least 30,000 nucleotides in length from a single primer terminus (82). Moreover, under these conditions the polymerase appears to be able to translocate through long stretches of duplex DNA (81). Thus, it is possible that fork movement during adenovirus DNA replication does not require a separate helicase activity. Rather, unwinding of the parental strands may be mediated solely by the Ad DNA polymerase and DBP, and the energy required for unwinding may be provided by the hydrolysis of deoxynucleoside triphosphates. This possibility is consistent with the observation that little, if any, ATP hydrolysis occurs during adenovirus DNA replication in vitro (81).

In the presence of the pTP, the adenovirus DNA polymerase, the DBP and NF-I nascent adenovirus DNA chains are elongated to only about 25% of full length (20). Synthesis of complete adenovirus DNA strands requires an additional cellular protein, nuclear factor II (20). Nuclear factor II from HeLa cells has a native molecular weight of approximately 30,000 and copurifies with a DNA topoisomerase activity. Human or calf thymus topoisomerase I (but not *E. coli* topoisomerase I) will substitute for nuclear factor II in the adenovirus DNA replication reaction. The precise function of nuclear factor II in adenovirus DNA chain elongation is not yet clear. Since the protein has no significant effect on the synthesis of nascent strands up to 9 kb in length, it is presumably required to overcome the inhibitory effects of some DNA structure that appears only after extensive DNA synthesis.

SV40 DNA REPLICATION

SV40 has proven to be an excellent model system for studying the mechanisms of cellular DNA replication (88–91). The viral genome consists of a circular duplex DNA molecule of about 5000 base pairs and contains one origin of DNA replication. SV40 DNA replication takes place in the nucleus of the host cell where the viral genome is complexed with histones to form a nucleoprotein structure (minichromosome) indistinguishable from cellular

chromatin. Since SV40 encodes only a single replication protein (T antigen), the virus makes extensive use of the cellular replication machinery. As a result there are many similarities between viral and cellular DNA replication. In both cases initiation of DNA synthesis results in the establishment of two replication forks that move in opposite directions. At each fork one of the two nascent strands (the leading strand) grows continuously, while the other strand (the lagging strand) grows discontinuously by joining together small (ca. 200 bp) segments of DNA that are independently initiated with RNA primers. Completion of replication occurs when two oppositely moving forks meet. In linear cellular chromosomes the two merging forks originate from adjacent origins, while in circular SV40 chromosomes they have a single origin.

Much has been learned about SV40 DNA replication from in vivo studies (see reviews in Refs. 88–91). However, the recent development of an efficient cell-free replication system has greatly accelerated progress in understanding the molecular mechanisms involved (92). An important dividend of the dissection of the cell-free system has been the identification and functional characterization of components of the cellular replication apparatus. Thus, this review focuses on in vitro studies.

Initiation of SV40 DNA Replication

THE ORIGIN OF DNA REPLICATION The SV40 origin of replication is a 64-base-pair segment of the viral genome that contains all of the nucleotide sequence elements that are required for initiation of viral DNA replication in vitro and in vivo (93–106). Careful genetic analysis of base substitution mutations has revealed that the origin is complex, consisting of at least three functionally distinct sequence domains (104–106). At the center of the origin are four copies of a pentomeric sequence motif (GAGGC) organized as an inverted repeat. This sequence element is recognized by the viral initiation protein, T antigen (101, 106–120). On one side of the T antigen–binding site is a 17-base-pair segment containing A/T base pairs (105). It is suspected that this is the initial site of strand opening during initiation of SV40 DNA replication. On the other side of the T antigen–binding site is a 15-base-pair imperfect palindrome of unknown function. All three sequence domains of the origin are required for SV40 DNA replication, and there is some evidence that the spacing between them is critical for origin function (104).

Although the 65-base-pair core origin region is sufficient to support the initiation of SV40 DNA replication, sequences outside of the core can significantly influence the efficiency of initiation. A second T antigen–binding site located adjacent to the core origin increases replication efficiency several-fold both in vivo and in vitro (97, 101–103, 121, 122). Of even greater importance are elements previously associated with the activation of SV40 transcription,

such as the SV40 enhancers or the binding sites for the transcriptional factor Sp-1. The presence of either of these sequence elements adjacent to the core origin increases the efficiency of DNA replication in vivo at least 10-fold (99–103, 121, 123). For maximal stimulation of SV40 DNA replication, the transcriptional elements must be relatively close to the core origin. Insertion of 180 base pairs between the core origin and the Sp-1 binding sites completely abolishes the effect (121, 124). As documented elsewhere in this review, the activation of DNA replication by enhancers and other transcriptional elements is not limited to SV40 and, in fact, appears to be a quite general feature of the replication of eukaryotic viruses. In the case of SV40, there is some evidence that the binding of transcriptional activator proteins affects replication indirectly by perturbing the local distribution of nucleosomes, so that the DNA in the adjacent core region is relatively nucleosome-free. This presumably facilitates the interactions of the core origin with T antigen and other initiation proteins. Direct analysis of SV40 chromatin isolated from infected cells has revealed that the core origin region is less likely to be packaged into a nucleosome than other regions of the viral genome (124–129). In addition, studies of viral mutants have demonstrated that the genetic determinants of the nucleosome exclusion effect reside in the SV40 enhancer elements and the Sp-1-binding sites (99, 130, 131). Thus, although other models have not been ruled out, it seems likely that the stimulatory effect of the transcriptional elements is due, at least in part, to effects on chromatin structure.

SV40 T ANTIGEN The SV40 T antigen is a virus-encoded phosphoprotein with a polypeptide molecular weight of 82,000 (88–91, 132, 133). The protein plays the central role in initiation of viral DNA replication. Binding studies suggest that a T antigen molecule binds to each of the four pentamer repeats in the origin to form an organized nucleoprotein structure that is competent for initiation (134, 135). The precise number of T antigen monomers involved in formation of the T antigen/origin complex is not known. At physiologic temperatures complex formation is greatly facilitated by ATP (136, 137). Binding of T antigen appears to cause significant changes in the local DNA structure of the origin. Chemical probes have revealed destabilization of the helix in the region of the imperfect 15-base-pair palindrome and a structural deformation of unknown character in the AT-rich region (138).

Recently, considerable progress has been made in understanding the role of T antigen in the initiation of SV40 DNA replication. This is in large measure due to the discovery that T antigen has an intrinsic helicase activity (139). Once it is bound to the origin, T antigen is capable of entering the duplex and catalyzing the ATP-dependent unwinding of the two DNA strands (140–142). Unwinding appears to be a critical step that establishes the replication forks

and generates the substrate that is required for the priming and elongation of nascent strands. The helicase activity of T antigen may also be involved in the elongation process itself (see below).

The unwinding reaction requires the presence of the AT-rich segment of the origin as well as the T antigen recognition site (143). Analysis of base substitution mutations in the AT-rich segment indicates that the precise nucleotide sequence of the segment, not just its base composition, is an important determinant of the efficiency of unwinding (105, 120, 143). One possible explanation for this observation is that T antigen makes sequence-specific contacts with the AT-rich domain during the course of the unwinding reaction. A second possibility is suggested by the finding that the AT domain induces a significant bend in the helix (105). Several single-base substitution mutations within the domain that reduce the efficiency of unwinding and replication also change the degree of net bending (105, 143). Thus, the conformation of the DNA in this domain may be critically important. For example, bending could contribute to the destabilization of the duplex in the AT domain or could facilitate the interaction of the domain with T antigen during entry or unwinding.

CELLULAR PROTEINS In addition to specific nucleotide sequence elements, the T antigen–mediated unwinding reaction requires accessory proteins contributed by the host cell. For example, a single-stranded DNA-binding protein is required to prevent reassociation of the single strands exposed during unwinding (140–142). It seems likely that this function is normally fulfilled by a recently identified cellular protein designated replication protein A (RP-A or RF-A) (144, 145). RP-A has been purified to homogeneity, and the purified protein is absolutely required for SV40 DNA replication in the reconstituted cell-free system (144, 145). The protein consists of three tightly associated subunits of 70 kd, 32 kd, and 14 kd. The largest subunit binds specifically to single-stranded DNA (146). Heterologous single-stranded DNA binding proteins, such as *E. coli* SSB, will substitute for RP-A in the unwinding reaction (140, 141); however, *E. coli* SSB cannot replace RP-A in the complete DNA replication reaction, indicating that RP-A must play other roles in DNA replication (141). A single-stranded DNA-binding activity associated with polypeptides of 72 kd and 76 kd has also been identified in protein isolated from HeLa cells (147). This activity stimulates SV40 DNA replication in vitro and may be related to RP-A. Although T antigen and RP-A appear to be the only proteins that are absolutely required for origin-dependent unwinding, recent evidence indicates that the efficiency of unwinding is increased significantly by a second cellular protein, designated RP-C (146). Although it seems likely that RP-C interacts with T antigen during the early stages of the reaction, its precise function is not yet clear.

Elongation of Nascent DNA Chains

Fractionation of the cell-free SV40 system has yielded considerable information on the proteins involved in the elongation of nascent SV40 DNA chains.

DNA POLYMERASE α Mammalian cells contain four distinguishable DNA polymerase activities designated α, β, γ, and δ (148, 149). Of these, DNA polymerase α and probably DNA polymerase δ are required for SV40 DNA replication (92, 146, 150–156). DNA polymerase α has long been considered to be the major replicative polymerase in animal cells and has been purified from a variety of sources by several laboratories (148, 150, 157). There is general agreement that the enzyme is composed of four distinct subunits (148, 157). The largest subunit (180 kd) contains the polymerase active site. The large subunit of the *Drosophila* DNA polymerase α has also been shown to harbor a cryptic 3'→5' exonuclease that serves a proofreading function during polymerization (158). The exonuclease activity is normally masked by a second subunit of the polymerase with a molecular weight of 70,000. The exonuclease activity has not yet been detected in enzymes from species other than *Drosophila,* but its presence seems likely given the apparent structural conservation of DNA polymerase α during evolution. The proofreading exonuclease activity is probably an important general feature of DNA polymerase α that contributes significantly to the fidelity of DNA replication. The smallest subunits of DNA polymerase α (50–60 kd) constitute a primase enzyme capable of synthesizing short RNA transcripts that can serve as primers for subsequent DNA chain elongation by the catalytic subunit (148, 157, 159, 160). The mammalian DNA polymerase α is not a highly processive enzyme, as less than 100 nucleotides are polymerized per binding event under the usual assay conditions (161–164).

There is evidence that DNA polymerase α can form a specific complex with the SV40 T antigen. Thus, certain monoclonal antibodies against T antigen (or DNA polymerase α) will coprecipitate the two proteins from cell extracts (165, 166). The specificity of this interaction may play some role in determining the host specificity of SV40. For example, it has been observed that preparations of human DNA polymerase α, but not murine DNA polymerase α, can activate T antigen–dependent DNA replication in extracts of murine cells that are normally defective in SV40 DNA replication (151). In both lytically infected and transformed cells, T antigen is also associated with a cellular protein of unknown function referred to as p53 (167–169). Recent experiments suggest that murine p53 competes with DNA polymerase α for binding to T antigen (166). The significance of this observation is not yet clear.

DNA POLYMERASE δ AND PCNA DNA polymerase δ was originally distinguished from DNA polymerase α because it contains a readily detectable 3'→5' exonuclease activity (170). The two polymerases also differ from one another in their template preferences, chromatographic properties, and sensitivities to various inhibitors (149, 170–174). Monoclonal antibodies against DNA polymerase α do not inhibit the activity of DNA polymerase δ, and DNA polymerase δ appears to lack an intrinsic primase activity. Thus, it appears likely that DNA polymerases α and δ are distinct molecular entities. However, since the structure of DNA polymerase δ has not been characterized in detail, it remains possible that the two polymerases share similar or identical subunits.

A 37-kd protein that dramatically increases the activity of DNA polymerase δ with template/primers containing long single-stranded regions was recently purified to homogeneity (175). Interestingly, the 37-kd protein proved to be identical to a previously described polypeptide found specifically in proliferating cells and referred to as proliferating cell nuclear antigen (PCNA) or cyclin (153, 154, 176). In the presence of PCNA, DNA polymerase δ is a highly processive enzyme capable of catalyzing the polymerization of at least 1000 nucleotides per binding event (164, 175). PCNA has no effect on the activity or processivity of DNA polymerase α (164).

It has been demonstrated by direct reconstitution that PCNA is required for efficient SV40 DNA replication in the cell-free system and is probably involved in DNA chain elongation (146, 152–154). In the absence of PCNA, initiation of DNA synthesis at the origin occurs, but only short nascent strands, containing a maximum of a few hundred nucleotides, are synthesized. The requirement for PCNA suggests the possibility that DNA polymerase δ may be involved in SV40 DNA replication, a view that is also supported by inhibitor studies. More direct evidence on this point has come from recent experiments suggesting that both DNA polymerase α and DNA polymerase δ are required to reconstitute efficient DNA replication in the SV40 cell-free system (146).

THE TWO POLYMERASE MODEL If both DNA polymerase α and DNA polymerase δ are involved in DNA replication, it would seem likely that they fulfill different functions. One can envision several possible ways that two polymerases could divide the labor of replication, but one particularly interesting possibility is that DNA polymerase δ serves as the leading strand polymerase and DNA polymerase α serves as the lagging strand polymerase (164). This model is consistent with the known biochemical properties of the two enzymes. The leading strand polymerase would be expected to be highly processive and would derive little benefit from an associated primase activity.

The lagging strand polymerase, on the other hand, would require only moderate processivity, but would benefit enormously from a tightly associated primase activity. The model is also supported by recent studies indicating that PCNA, the accessory factor for DNA polymerase δ, is required for leading strand synthesis in the reconstituted SV40 DNA replication system (177).

TOPOISOMERASES The roles of DNA topoisomerases in SV40 DNA replication have been investigated by depleting the cell-free system of topoisomerase activities with specific antibodies and then reconstituting with the purified enzymes (178). These experiments have demonstrated that DNA topoisomerase activity is absolutely required for SV40 DNA replication and have established two distinct roles for DNA topoisomerases in the replication process. One role is to act as a swivel to relieve superhelical tension that would otherwise hinder the unwinding of the parental strands as the replication forks advance. Either of the two known mammalian DNA topoisomerases, topoisomerase I or topoisomerase II, can provide this function. The second role is to mediate the separation of the newly synthesized daughter duplexes at the completion of DNA replication. In general, the links between the parental strands are not completely removed prior to termination of SV40 DNA synthesis, so the immediate products of replication consist of two circular DNA molecules that are multiply intertwined (179). The final act of replication is the segregation of these intertwined daughter molecules into two separate unlinked molecules. This reaction is catalyzed by topoisomerase II. It is of interest to note that genetic experiments in yeast indicate that the topoisomerases probably play these same two roles during the replication and segregation of cellular chromosomes (180–182).

HELICASE Unwinding of the parental DNA ahead of the advancing replication fork presumably requires the action of a helicase. The identity of the helicase activity required for fork movement during SV40 DNA replication has not been established with certainty. One reasonable possibility is that T antigen fulfills this function in addition to its role in unwinding the origin region prior to initiation of DNA synthesis (183). In the presence of a single-stranded DNA-binding protein, the intrinsic helicase activity of T antigen is capable of unwinding long segments of duplex DNA in a highly processive manner (142, 184, 185). Moreover, it has been reported that monoclonal antibodies that inhibit the helicase activity of T antigen also inhibit ongoing chain elongation in a subcellular SV40 DNA replication system (183). Studies with model substrates indicate that the T antigen helicase translocates in the 3' to 5' direction on single DNA strands (184, 185). Thus, if T antigen is the helicase responsible for fork involvement, it would be expected to move along the leading strand. In contrast, all of the

prokaryotic helicases that are known to be directly involved in DNA replication move along the lagging strand (185).

It remains possible that a cellular DNA helicase, rather than T antigen, is responsible for unwinding the parental strands at SV40 replication forks. While helicase activities have been identified in extracts of eukaryotic cells, it is not yet clear whether any of these activities are involved in SV40 (or cellular) DNA replication (186–188). It has also been reported that DNA polymerase δ is capable of some degree of strand displacement during DNA synthesis, suggesting the possibility that a separate helicase activity may not be required (163). Alternatively, the helicase activity required for chromosomal replication may reside in an as-yet-unidentified cellular protein.

HERPES SIMPLEX VIRUS DNA REPLICATION

The herpesviruses are DNA-containing viruses infecting a variety of animal species. Of the more than 80 different herpesviruses that have been isolated, the most extensively characterized are the human viruses herpes simplex virus type 1 (HSV-1) and the closely related virus, HSV-2 (collectively, HSV). HSV replicates lytically in epithelial cells in infected individuals and in a wide variety of cultured mammalian cells. The molecular biology of HSV has been studied extensively and a great deal is known about the structure of the genome, and the arrangement and regulated expression of viral genes (reviewed in Refs. 189–191). HSV DNA is a linear double-stranded molecule about 153 kb in size. The genome consists of two components, L and S, each of which is flanked by inverted repeat sequences (see Figure 2). Although the inverted repeats flanking the L and S components are different, they share a small terminal sequence (termed the "a sequence") so that HSV DNA is terminally redundant. During the course of viral replication, the two components invert relative to each other, so that purified viral DNA consists of an equimolar population of four isomers that differ in the relative orientations of L and S. It is clear that isomerization is not an obligatory feature of HSV DNA replication. Mutant viruses containing a deletion of sequences at the L/S junction are frozen in a single isomeric arrangement but nevertheless are viable and replicate DNA at normal levels (192). The HSV genome has now been completely sequenced (193). Analysis of the sequence suggests that HSV encodes a minimum of 72 proteins. Splicing of HSV genes is very uncommon, a fact that has greatly simplified the genetic analysis of this virus (189–191, 193).

Like all of the herpesviruses, HSV has the capacity to remain latent in an infected host. In the case of HSV, the cells that harbor the latent viral genome are neurons in sensory ganglia (reviewed in Refs. 194, 195). It is not yet known whether HSV, like some of the other herpesviruses that are latent in

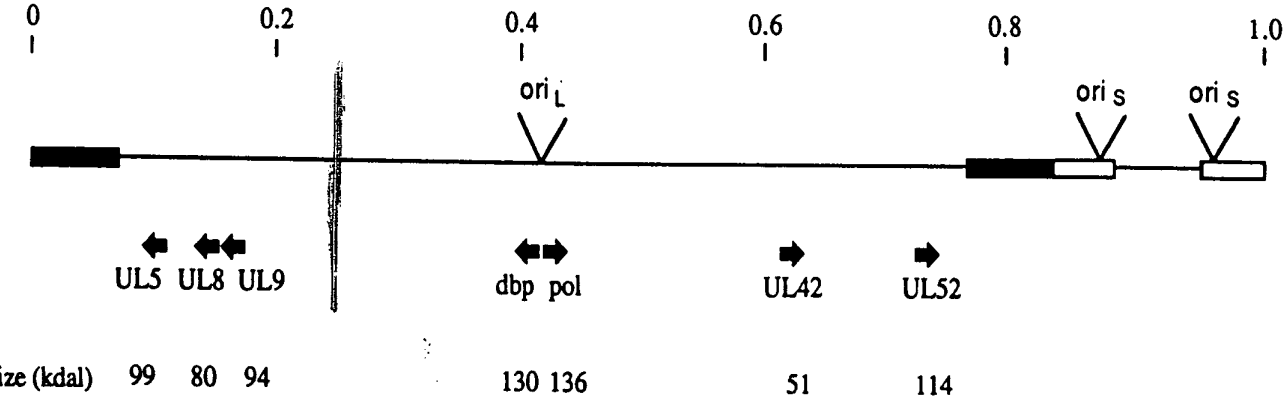

Figure 2 Organization of the herpes simplex virus genome. Unique regions of the genome are indicated by thin lines. Filled boxes and open boxes indicate the inverted repeats flanking the long unique region and short unique region, respectively. Arrows indicate open reading frames (ORFs) encoding proteins involved in DNA replication.

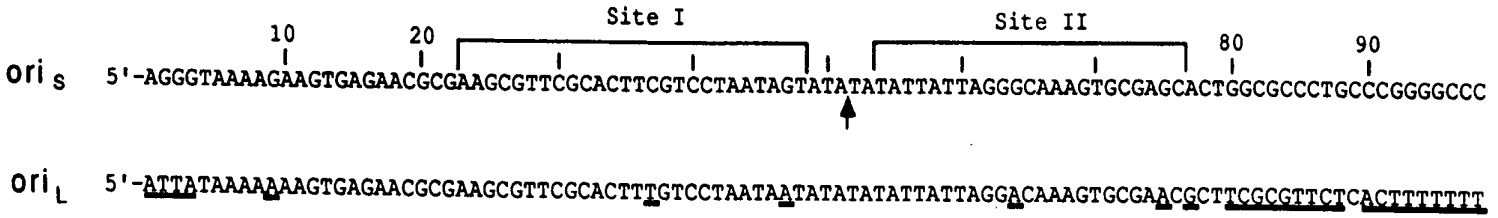

Figure 3 Sequence of the herpes simplex virus origins of DNA replication. The nucleotides in ori$_L$ that differ from those in ori$_S$ are underlined.

dividing cells, has a regulated mode of DNA replication in the latent phase of infection; since neurons do not divide, however, there is no necessity for such a system. In this review we focus on the only documented mode of HSV DNA synthesis: the DNA synthesis that occurs during lytic growth of the virus.

In Vivo Studies

Studies of the overall mechanism of the HSV DNA replication in vivo have been hampered by the large size of the viral genome, but several important facts have emerged from analyses of the properties of replication intermediates and from electron microscopic examination of viral DNA isolated from infected cells (196–198). Replication intermediates have two characteristic properties. First, DNA pulse-labeled in vivo with 3H-thymidine sediments more rapidly than unit length viral DNA (196). Second, pulse-labeled DNA is "endless"; i.e. the molecular termini of mature viral DNA are fused together (197, 198). On the basis of these observations, it has been proposed that parental linear viral DNA is circularized shortly after entry into the host cell, and that replication takes place predominantly by a rolling circle mechanism, generating linear concatamers of tandemly repeated viral genomes (197). Although this remains an attractive hypothesis, it is largely untested.

Circularization of the incoming viral genome prior to the onset of DNA synthesis is certainly the simplest explanation for the complete lack of genomic termini in replicating DNA. In fact, unit-length circular genomes have been observed by electron microscopic analysis of viral DNA in infected cells (196). A comparison of the structure of the termini of mature DNA and L/S junction sequences suggests that circularization takes place by the direct ligation of the ends of linear viral DNA (199).

The mechanism(s) responsible for the very rapid sedimentation properties of replicating viral DNA is less clear. Certainly rolling circle–type replication would account for such intermediates. Other means of generating fast-sedimenting DNA, however, are plausible. For example, it is possible that replication takes place by a "Cairns" type mechanism and that segregation of daughter molecules is slow (198). Alternatively, daughter molecules may be rapidly joined together by homologous recombination. There is some indirect evidence that is consistent with the latter possibility. First, the structure of replicating viral DNA may not be simple. Electron microscopic studies have suggested that much of the viral DNA in infected cells is composed of complex networks (196). Second, several studies have shown that HSV DNA in infected cells undergoes high levels of homologous recombination (200–202). The rate of recombination has been estimated at about 1–3% per kb per infectious cycle (200). This high rate of recombination is closely linked to DNA synthesis (203). By analogy with bacteriophage T4, which produces replication intermediates with very similar properties (204, 205), it is possible

that the networks are formed by a combination of DNA synthesis and recombination and that resolution of recombinational intermediates is a slow step. Clearly, this overall scheme alone could account for the sedimentation properties of pulse-labeled DNA, although recombination and rolling circle replication are, of course, not mutually exclusive. Recently developed techniques such as pulse-field gel elecrotrophoresis may provide a means to reevaluate some of these questions.

Replication Origins

The existence of *cis*-acting replication origins was first inferred from the structure of defective genomes (206–212), which arise during serial passage of HSV at high multiplicities of infection. Individual isolates of such defective genomes have several common features. They all consist of many tandemly repeated identical copies of small segments of viral DNA; the sequences present in a single repeat unit are not necessarily colinear with a single segment of viral DNA. Two classes of defectives were recognized (206–214). Class I defectives contain sequences from the short inverted repeat segment of the viral genome including the "a" sequence. Class II defectives contain sequences from near the middle of the long unique region, as well as the "a" sequence. It was proposed that the sequences present in defective genomes contain two *cis*-acting signals: an origin of replication, many copies of which would account for the selective advantage of defectives; and the "a" sequence, required for the formation of genomic termini from "endless" intermediates and for packaging of the DNA into infectious virions (212, 215, 216, 218, 219). This general picture of defectives has now been verified in several laboratories. Both the origin sequences and the cleavage/packing site have been cloned from the wild-type genome and analyzed in some detail. The analyses of origin sequences will be discussed below; since the cleavage/packaging signal is not required for DNA replication per se, it will not be discussed further. The interested reader is referred to several recent publications (221–223).

As implied from the existence of two classes of defective genomes, HSV contains two distinct origin sequences (212). As mentioned, both of the sequences have been cloned from the wild-type viral genome (211–219, 225–227). Plasmids containing either of these origin sequences are amplified when introduced into HSV-infected cells by transfection, and this transient plasmid amplification assay has formed the basis for most of the functional analyses of origin sequences. The origin present in class I–defective genomes has been designated ori_S (216, 218, 219). Since it is located within the inverted repeat sequence of the short component of viral DNA, there are two copies of ori_S in the HSV genome. The origin contained in class II–defective genomes, designated ori_L, is located in the middle of the long unique com-

ponent of viral DNA (209, 214). The sequences of ori_S and ori_L are closely related (224, 227; see Figure 3). Both contain a rather extensive inverted repeat sequence, the central 18 base pairs of which are exclusively AT base pairs. The inverted repeat of ori_L is considerably longer than that of ori_S; for this reason, plasmids containing ori_L, but not ori_S, are highly unstable in *E. coli*. Therefore, most of the functional analyses of HSV origin sequences have been done with ori_S. As described below, the minimum sequences required for the function of ori_S correspond well to the region of highest similarity with ori_L (228). Thus it is likely that ori_S and ori_L are functionally equivalent, although direct evidence for this supposition is currently lacking. The functional significance of three separate origins of replication in the HSV genome is not clear. Mutant viruses lacking ori_L or one copy of ori_S have been isolated and have no obvious defect in growth (229, 230). Attempts to construct a mutant virus lacking both copies of ori_S have been unsuccessful to date; this seems to imply that virus replication requires at least one copy of ori_S or, alternatively, at least two origin sequences, whether they be ori_L or ori_S. Additional genetic experiments will be required to answer these questions.

Several laboratories have carried out deletion analyses of plasmids containing ori_S, with somewhat conflicting results (218–220, 228, 231). In the most extensive recent study (228), the left-hand boundary of the minimal or core ori_S sequence was shown to lie between nucleotides 5 and 11 in the sequence shown in Figure 3, about 20 nucleotides to the left of the left arm of the palindrome. The right-hand boundary was located between nucleotides 74 and 77, corresponding almost precisely to the outer border of the right arm of the central palindrome. As detailed below, the two arms of the palindrome contain binding sites for a viral-encoded protein required for DNA synthesis, UL9. Deletion of the central AT-rich component of the palindrome completely eliminated DNA replication, as did replacement of the central AT region with an equal number of GC base pairs. Insertion of AT base pairs at the center of symmetry had an oscillating effect on function, with a periodicity of approximately 10 base pairs. Taken together with studies on the interaction between the origin and the HSV origin binding protein (see below), these results suggest that that ori_S is composed of at least four distinct domains: the two arms of the palindrome, which serve as binding sites for UL9; the central AT-rich region, which serves as a spacer between the protein-binding sites and, by analogy with other origin sequences, potentially as a site of protein-induced unwinding; and the 20 or so base pairs to the left of the palindrome, to which no precise function has as yet been ascribed. As noted earlier, ori_L is very similar to ori_S throughout this core region (see Figure 3). Moreover, because of the greater degree of symmetry in ori_L, the left-most domain of ori_S is represented twice in ori_L.

While there is general agreement among several laboratories that the left

arm of the palindrome and the AT-rich region at the center of the palindrome are essential components of ori_S, there are conflicting results on the requirement for the right arm of the palindrome. In contrast to the results of Lockshon & Galloway (228), Deb & Doelberg (231) have reported that a plasmid completely lacking the right arm replicates equally as well as a control plasmid containing the intact origin. Studies on the origin of another alpha herpesvirus, varicella zoster virus (VZV), provide some additional support for this view (232). HSV-infected cells support the replication of plasmids containing the VZV origin of replication at a level about 10% that of plasmids containing ori_S. The VZV origin contains sequences homologous to the left arm of the HSV palindrome and a somewhat expanded AT-rich region to the right, but no sequences corresponding to the right arm of the ori_S palindrome. Clearly, more work needs to be done to resolve the discrepancies in the identification of the minimal HSV origin sequence.

The minimal HSV origin sequence, whether it includes the right arm of the origin palindrome or not, does not contain any obvious transcriptional regulatory sequences. Both ori_S and ori_L are located between divergently transcribed genes (233–237), but there is no evidence that either the promoters for these genes or any upstream regulatory elements have any effect on origin function. There is evidence that sequences flanking the core origin sequence do have a modest stimulatory effect on the extent of DNA replication in the plasmid amplification assay (218, 219, 228), but whether or not these stimulatory sequences are related in any way to transcriptional enhancer sequences, as is the case for other origins, remains to be determined. There is a transcript in HSV-infected cells that extends through ori_S (238). Neither the 5' nor 3' end of this transcript is located within sequences that have an effect on origin function. It has been suggested that this transcript plays a role in regulating DNA replication, but no evidence supports this speculation as yet.

Clearly, many questions remain concerning the HSV origins. By analogy with other better characterized systems, it seems likely that DNA replication begins at one or more of the origin sequences. There is no information as yet, however, on the molecular events by which HSV DNA replication is initiated. It seems likely that further insight into this process will await the development of a soluble, origin-dependent in vitro system.

Genetics of Viral DNA Replication

A large number of conditionally lethal mutants of HSV have been isolated and characterized over the past decade, and many of these mutants have defects in DNA synthesis (reviewed in Ref. 239). Some mutations, such as those occurring within the gene encoding an immediate early transcriptional regulatory protein (240, 241), affect viral DNA synthesis indirectly, but many mutants apparently have defects directly affecting DNA replication. There are

seven complementation groups of such mutants now known (241–254), and for reasons that will be discussed below, it is unlikely that any more will be found.

The complete set of viral genes that are required for DNA replication were identified by means of a transient complementation assay in which cloned segments of HSV DNA were tested for the ability to support the replication of a cotransfected plasmid containing ori_S or ori_L (255). Seven genes were found to be both necessary and sufficient for origin-dependent DNA synthesis (256; see Figure 2). Detailed mapping of available ts mutants with clear DNA^- phenotypes has shown that all such mutants have defects in one of the seven genes identified using the transient assay system (241–249, 252, 254). In addition, viruses containing targeted null mutations (insertions or deletions) in five of these seven genes have now been isolated (251–253; P. A. Schaffer, personal communication; D. M. Knipe, personal communication). These viruses, which can be propagated in complementing cell lines expressing the wild-type gene, all fail to synthesize viral DNA in noncomplementing host cells. Thus, there is complete correspondence between the genes required for DNA synthesis in the transient system and the genes identified by mutational analysis to be directly involved in viral DNA replication.

The functions of the products of most of these genes are either unknown or only recently identified (see below). Thus, the use of virus mutants to analyze the properties of replication proteins has been limited. The exceptions are the genes encoding a DNA polymerase and a single-stranded DNA-binding protein, which have been known for some time and have been the subject of genetic analysis in several labs. Since, as mentioned above, directed mutagenesis of essential HSV genes is now possible, a great deal more information of this sort should be forthcoming in the near future.

Several temperature-sensitive mutants with amino-acid substitutions in the HSV DNA polymerase have been characterized (241–245). All of these mutants fail to synthesize viral DNA at the restrictive temperature. Since the DNA polymerase is the target of several antiviral drugs (deoxynucleotide and pyrophosphate analogues), it has also been possible to isolate virus mutants in which the encoded polymerase has altered sensitivities to these drugs (258–265). All such mutations that have been mapped carefully occur in the carboxy-terminal half of the DNA polymerase (from amino acid number 397 to 961). It has been proposed that these amino acids contain the nucleotide-binding domain of the DNA polymerase (266, 267, 267a). In this context it is also of interest that several regions of homology among the amino acid sequences of many DNA polymerases, both eukaryotic and prokaryotic, have been noted (266–268). Most of these regions of homology occur within the sequence of the HSV polymerase in the region proposed as the nucleotide-binding domain. Several polymerase mutants selected for resistance to the

inhibitor phosphonoacetic acid were shown to also have an anti-mutator phenotype (269). The biochemical properties of the mutant polymerases that account for this phenotype have not yet been determined.

The other HSV DNA replication gene that has been extensively analyzed is that encoding a single-stranded DNA-binding protein (infected-cell polypeptide 8, or ICP8). Temperature-sensitive mutants with amino acid substitutions in the ICP8 coding sequence fail to synthesize viral DNA at the restrictive temperature (246–248). Some mutant ICP8 proteins are defective in nuclear localization, while others have been shown to have a defect in DNA binding (270–273). Fine mapping of mutants that encode ICP8 proteins that do localize to the nucleus has revealed that at least some of the residues between 346 and 450 are necessary for DNA binding (273). Several ts mutants with defects in ICP8 have also been shown to display altered sensitivities (at the permissive temperature) to drugs that inhibit the viral DNA polymerase (274). Finally, some ICP8 ts mutants overexpress certain late genes at the nonpermissive temperature (275, 276). Thus it is possible that ICP8 plays a role in the regulation of viral gene expression in addition to its role in viral DNA replication. It is not yet known whether such a regulatory function is related to the ability of ICP8 to bind to single- and double-standed nucleic acids, or whether it represents a completely unrelated activity.

In ending this section on the genetics of viral DNA replication, it is important to note that although HSV contains only seven genes that are required for viral DNA replication in cultured cells, the virus clearly encodes several other proteins that are also likely to be involved in DNA metabolism. These include a thymidine kinase (277), a ribonucleotide reductase (278, 279), a dUTPase (280), a uracil-N-glycosidase (281), and a nuclease (282, 283). Genetic studies have shown that while mutations in these genes have only minor or no effects on viral DNA synthesis in infected cells in culture, they may cause profound defects in the ability of the virus to replicate following experimental infection of animals (284–286; S. K. Weller, personal communication). It is likely, therefore, that rapidly dividing cultured cells provide many functions that are lacking in the cells encountered by the virus during a natural infection. Whether host cells provide any functions necessary for DNA replication that are not encoded by the virus remains to be determined.

Analysis of the Sequences of the HSV-1 Replication Proteins

The complete sequences of two other human herpesviruses in addition to HSV have been determined: VZV, like HSV an alphaherpesvirus (287); and EBV, a member of the more distantly related herpesvirus subgroup, the gammaherpesviruses (288). These viruses have biological properties that differ markedly from HSV, but the available evidence suggests that DNA replica-

tion during the lytic growth of these and all herpesviruses occurs by fundamentally the same mechanism. Thus it is of some interest to search the genomes of the less-well characterized herpesviruses for homologues to the HSV genes that are essential for DNA synthesis. A clear homologue of each of the seven HSV genes is present in the genome of VZV (257). Counterparts of four HSV genes (pol, ICP8, UL5, and UL52) with clear sequence similarity are present in the genome of the more widely diverged virus, EBV (257). Counterparts of the three remaining HSV genes can be proposed on the basis of genomic location alone, but for two of these, UL8 and UL9, they are markedly different in size. The functional significance of these similarities (or lack thereof) is not yet known. The lack of a clear counterpart in EBV of the HSV gene UL9, which encodes an origin-specific DNA-binding protein, is consistent with the fact that the lytic origin of EBV is not discernibly similar to the HSV origins (288a). It seems likely that as more information becomes available concerning the biochemical functions of the HSV replication proteins that these sequence comparisons will provide some useful insights into structure-function relationships. Conversely, knowledge of the function of the HSV genes should prove extremely useful in the analysis of the replication of the genomes of these other viruses.

The predicted sequences of the HSV DNA replication proteins have been examined for the presence of several consensus elements (257). Three of the proteins were found to contain a motif associated with nucleotide binding sites (289): the DNA polymerase, UL5, and UL9. This motif was also conserved in the VZV homologues of these genes and in the EBV homologues of the DNA polymerase and UL5. More recently, UL5 (and the VZV and EBV homologues of UL5) has been shown to contain several additional regions of similarity with a large group of proteins that all have helicase function (289a, 289b, 289c). It seems likely, therefore, that UL5 is a helicase.

Biochemical Analysis of Replication Proteins

The complete characterization of HSV DNA replication will ultimately depend on the development of a soluble, origin-dependent in vitro system. No such system has yet been described, but it has been possible to carry out some biochemical analyses of selected replication proteins. By analogy with other, better characterized systems, it is possible to predict certain biochemical activities that might be involved in HSV DNA replication. Extracts of infected cells can thus be analyzed for the presence of virus-induced proteins with these activities, and if found, the proteins can be purified and further characterized. In addition, since the complete set of viral genes required for DNA synthesis is now known and the products of these genes identified using specific antisera (290), it is possible to purify these proteins without any prior knowledge or assumptions concerning their functions. The purified proteins can then be assayed for pertinent biochemical activities.

DNA POLYMERASE Extracts of HSV-infected cells contain a novel DNA polymerase activity (283, 291, 292). This activity is readily distinguished from the host DNA polymerases on the basis of its sensitivity to various inhibitors and by the fact that it is stimulated, rather than inhibited, by moderate concentrations of salt (292–295). As mentioned earlier, analysis of ts and drug-resistant mutants has clearly shown this enzyme to be virus-encoded. The HSV DNA polymerase has been extensively purified in several laboratories. As is the case with many other DNA polymerases, it has an intrinsic 3'-5' exonuclease activity that probably serves a proofreading function to increase the fidelity of DNA synthesis (294–296). The most highly purified preparations of the enzyme consist predominantly of a monomer of a single polypeptide chain of ~140 kd in size (294, 295), in good agreement with the size of the product of the polymerase gene predicted from DNA sequence analysis (266, 297). An unusual property of the HSV polymerase is that it is highly processive in the absence of accessory factors (295).

It is clear that the 140-kd polypeptide product of the polymerase gene itself has catalytic activity on simple primer templates such as activated DNA. As mentioned above, the most highly purified preparations of the polymerase do not contain any other polypeptide in stoichiometric amounts (but see below). In addition, catalytically active polymerase has been expressed by in vitro transcription/translation (295a) in yeast (295b) and in insect cells using a recombinant baculovirus (D. Coen, A. Marcey, P. Olivo, personal communication). It is not yet clear, however, whether there are other forms of the polymerase that contain additional accessory factors that increase the efficiency of the polymerase or modify its activity in some other way. It has been reported that the 140-kd polypeptide of HSV-2 co-purified with a 55-kd polypeptide (298). This 55-kd polypeptide has since been shown to be the product of the *UL42* gene, one of the HSV genes that is essential for DNA synthesis (see below); the effect of the UL42 protein on the activity of the polymerase, however, has not yet been determined.

SINGLE-STRANDED DNA-BINDING PROTEIN ICP8 was recognized several years ago as an abundant HSV-induced protein of about 130 kd present in infected cells but not in virions (299, 300). ICP8 was shown to bind tightly to single-stranded DNA cellulose columns (301–305). Since, as indicated earlier, genetic evidence indicates that this protein is required for viral DNA synthesis, it seems reasonable to assume that the function of ICP8 is analogous to that of the gene 32 protein of bacteriophage T4 and the SSB protein of *E. coli:* namely, to bind to the single-stranded DNA formed at a replication fork by the unwinding of the parental duplex DNA, and to facilitate the use of these strands as templates for DNA polymerase. ICP8 in fact has many of the properties that are characteristic of this class of replication proteins: it binds

more tightly to single-stranded DNA than to double-stranded DNA (301–306); binding to single-stranded DNA is cooperative and is independent of sequence (305). It has been difficult, however, to obtain direct biochemical evidence that single-stranded DNA bound to ICP8 is a better template for the HSV DNA polymerase than naked DNA. It has been reported that purified ICP8 has a small (no greater than twofold) stimulatory effect on the activity of purified HSV DNA polymerase using activated DNA as template (307). Surprisingly, however, ICP8 was shown to inhibit the activity of the polymerase on a long single-stranded DNA template (306). Obviously, a great deal more work needs to be done concerning the function of ICP8 and its interaction with other replication proteins.

The isolation several years ago of a monoclonal antibody against ICP8 (308) stimulated a detailed analysis of its intracellular localization (272, 309, 310). As expected from its essential role in DNA replication, ICP8 is localized predominantly in the nucleus (309–311). Under conditions in which viral DNA synthesis is inhibited, ICP8 is located in small discrete foci that contain newly replicated host-cell DNA (272, 272a). During DNA synthesis, ICP8 was found to move from these small foci to larger, more globular areas that are the sites for viral DNA synthesis (272). It has been suggested that one function of ICP8 may be to organize the structure and composition of these different compartments (272a). Whether all the viral replication proteins are found at these sites remains to be determined.

ORIGIN-BINDING PROTEIN A protein that binds specifically to the HSV origins of DNA replication has been identified in extracts of HSV-infected cells (312). This protein has now been purified to near homogeneity by site affinity chromatography (313). It consists of a single major polypeptide of about 83 kd. The origin-binding protein has recently been shown to be the product of the HSV gene UL9, one of the seven genes that are essential for DNA replication. The UL9 protein was expressed in insect cells using a baculovirus expression system, and the recombinant UL9 protein was shown to interact with ori_S in a manner indistinguishable from that observed with the origin-binding protein purified from HSV-infected cells (314). Although the size of the UL9 protein predicted from DNA sequence analysis is 94 kd (257), the observed size of the protein expressed both by the recombinant baculovirus and in HSV-infected cells is 83 kd (313, 314). Sedimentation analysis of the purified recombinant protein suggests that it is a dimer in solution (M. D. Challberg, unpublished).

The purified UL9 protein binds to ori_S at two nearly identical sites, located on each arm of a palindrome (313, 314). Filter binding studies with synthetic double-stranded oligonucleotides corresponding to the two sites have shown that the intrinsic affinity for the site on the left arm is about 10 times greater

than for the site on the right arm (313). Methylation interference experiments and a comparison of different binding sites suggest that the recognition sequence for UL9 is contained within the eight-base-pair sequence GTTCGCAC (315; M. D. Challberg, unpublished). It has been proposed (315) that the recognition sequence is GT (T/G)CG, which is contained twice within the eight-base-pair recognition domain as inverted repeats that share a two-base overlap. There is as yet no direct evidence in support of this proposal. It is consistent with the fact that purified UL9 does form a stable dimer, and with the fact that UL9 has a lower affinity for the binding site on the right arm of the ori$_S$ palindrome, in which one of the inverted pentamers differs from the canonical sequence.

Although the intrinsic affinity of the binding sites for UL9 on the two arms of the ori$_S$ palindrome differ, DNase I footprint analysis of the interaction of purified UL9 with the complete ori$_S$ sequence suggests that the affinity of UL9 for the two sites is equal (313, 314). Moreover, deletion of the UL9-binding site on the right arm of the palindrome reduces the affinity of UL9 for the binding site on the left arm (M. D. Challberg, unpublished). These results suggest that there is a cooperative interaction between the protein bound at the two sites. It is not yet known whether this cooperativity has any functional significance, although the reported effect of insertions into the AT-rich region between the two UL9-binding sites is intriguing. As mentioned earlier, a series of mutant origins (228) have been constructed in which n copies of the AT dinucleotide were inserted into the center of the AT-rich region of ori$_S$. As n increases from 0 to 8, replication first sharply decreases to a minimum at $n=3$, then rises to a maximum at $n=5$ or 6, then decreases again. It is possible that this oscillation in activity reflects a requirement for the UL9 protein bound on each arm of the palindrome to be located on the same side of the DNA helix. This arrangement may be necessary to accommodate critical UL9-UL9 interactions. However, in view of the report that the right arm of the ori$_S$ palindrome has no effect at all on origin function (231; see above), this question bears further investigation.

There is now good evidence to indicate that the carboxy-terminal portion of the UL9 protein contains the DNA-binding domain. The carboxy-terminal 37 kd of UL9 has been expressed in *E. coli* as a fusion protein. This truncated UL9 protein still binds specifically to DNA fragments containing ori$_S$ (N. Stow, personal communication). There is also preliminary evidence that a truncated form of UL9 (of unknown size) containing the carboxy terminus and retaining ori$_S$-binding activity can be isolated from HSV-infected cells (N. Stow, personal communication; A. Koff, P. Tegtmeyer, personal communication). It is not yet known whether this truncated form has any biological function.

The role of UL9 binding in HSV DNA replication is not known. By analogy with other prokaryotic and eukaryotic replication origin recognition

proteins, the binding of UL9 to ori_S and ori_L may initiate the assembly of a multiprotein replication complex. Alternatively, or additionally, UL9 may be involved in unwinding the two parental strands at the origin as a prelude to the initiation of daughter strand synthesis. This latter possibility is strengthened by the observation that the predicted amino acid sequence of UL9 contains a consensus ATP-binding sequence similar to that found in the SV40 T-antigen and the *E. coli* dna A protein (M. D. Challberg, unpublished). There is no convincing evidence as yet, however, that the UL9 protein binds or hydrolyzes ATP, or is capable of unwinding DNA at the HSV origins. Since the protein can now be produced in large quantities, the answer to some of these questions should be forthcoming.

HELICASE/PRIMASE Infection of cells with HSV induces novel helicase and primase activities (316, 317). Recently, these two activities have been purified to homogeneity (J. Crute, I. R. Lehman, personal communication); both helicase and primase activities are components of a three-subunit enzyme composed of the products of the UL5, UL8, and UL52 genes. The helicase can utilize either ATP or GTP as a cofactor for unwinding. The activity of the helicase on model substrates suggests that it moves in the 5' to 3' direction on the strand to which it is bound. Thus, this enzyme may prime lagging strand synthesis as it unwinds DNA at a replication fork. The activities of the component polypeptides of the complex have not yet been determined, although as mentioned above, UL5 contains several sequence motifs that are shared by helicases.

DOUBLE-STRANDED DNA-BINDING PROTEIN As mentioned above, purified preparations of the HSV-2 DNA polymerase were reported to contain two major polypeptides: the 140-kd product of the polymerase gene, and a 55-kd protein (294). It has since been demonstrated that this protein is the product of the UL42 gene (318–320), which genetic experiments have shown to be required for viral DNA synthesis (254, 256, 257). The UL42 protein has now been purified from HSV-1-infected cells in several laboratories, but its function remains obscure (320; C. Wu, M. D. Challberg, unpublished). As indicated earlier, this protein is not required for the catalytic activity of the HSV DNA polymerase. Immunoaffinity purification of the UL42 protein has provided evidence that there is an interaction between UL42 and the polymerase (320). The functional significance of this interaction remains to be determined. The UL42 protein binds strongly in a sequence-independent fashion to double-stranded DNA (320; C. Wu, M. D. Challberg, unpublished). The nature of this binding and its effects on DNA structure have not been detailed. It is not known whether DNA binding is an essential component of the function of UL42 in DNA synthesis.

BOVINE PAPILLOMAVIRUS DNA REPLICATION

The papillomaviruses are a group of small DNA-containing viruses that are associated with epithelial tumors in a variety of animal species (reviewed in 321–324). Although most of the cells in such tumors contain many copies of the viral genome in a latent state, the tumor cells contain no infectious virus; productive infection by these viruses appears to be confined to terminally differentiated keratinocytes. It has not yet been possible to propagate any of the papillomaviruses in a cell culture system. A subset of the papillomaviruses, however, of which bovine papillomavirus type 1(BPV-1) is the most extensively studied, also induce fibropapillomas in their natural hosts, and this subset readily transforms established rodent fibroblast cell lines in culture (325, 326). As in the case of naturally occurring tumors, transformed rodent cells contain the viral genetic information in a latent state. Only a subset of viral genes are expressed in transformed cells (327–329), and no infectious virus is produced. Most of the information on the molecular biology of the papillomaviruses has come from a study of this model in vitro cell culture system.

The BPV-1 genome is a covalently closed, circular, double-stranded DNA molecule of 7945 base pairs (330, 331). All of the open reading frames (ORFs) of at least 400 base pairs are located on one strand, and all of the mRNA species detectable both in transformed cells and in productively infected bovine fibropapillomas are homologous to that same strand (327–329). A 5.4-kb subgenomic fragment of BPV-1 DNA is sufficient for transformation of rodent cells in vitro (332). Within the transforming fragment there are eight open reading frames (E1 through E8) contained within a 4.5-kb segment (330, 331). On the 5' side of these ORFs there is an approximately 1-kb segment that contains no large open reading frames. This segment (the long control region, or LCR; also referred to as the upstream regulatory region) appears to contain a number of *cis*-acting regulatory elements, including several transcriptional promoters (328, 333), a transcriptional enhancer that is activated by one of the E2 gene products (334, 335), and the origin of DNA replication (333, 336, 337; see below).

Although the arrangement of ORFs deduced from DNA sequence analysis has provided a useful starting point for the analysis of viral gene products, there is clear genetic and biochemical evidence to suggest that the number and structure of viral proteins cannot be predicted solely from the DNA sequence of the genome. Analysis of viral mRNAs by electron microscopy and cDNA cloning has revealed a number of mRNAs formed by complex splicing patterns (328, 329). Thus, some viral proteins may correspond to a single ORF, others to only a portion of a single ORF, and yet others to combinations of ORFs. It has been possible to predict the primary sequence of some viral proteins from sequence analysis of cloned cDNAs. In addition, segments of

several ORFs have been expressed in bacteria and the resulting proteins used to produce specific antisera for the direct analysis of viral proteins in transformed cells (338–340). Several viral proteins have now been identified, but much remains to be learned.

In cells transformed by BPV or cloned BPV DNA, the viral DNA is maintained extrachromosomally as a stable multicopy plasmid in the cell nucleus (341). As detailed below, the available evidence suggests that only a single viral gene product is directly involved in viral DNA synthesis; it seems likely, therefore, that viral DNA replication is carried out largely by host cell proteins. Moreover, the copy number of BPV plasmids appears to be tightly controlled, and there is evidence that each BPV genome replicates once and only once per cell cycle (342). Thus, BPV appears to represent a useful model for analyzing the mechanisms involved in regulating DNA replication in higher eukaryotes.

BPV DNA Replication In Vivo

The site at which DNA replication initiates has been determined from studies of the structure of replicative intermediates (336). Covalently closed viral DNA was isolated from hamster cells transformed by wild-type BPV and analyzed by electron microscopy. Circular molecules with two forks and no free ends were observed. By measuring molecules cleaved with various single-cut restriction enzymes, the position of the replication eye in such intermediates was localized to map position 6940 ± 5%. Thus, replication of latent BPV genomes initiates within the LCR and proceeds by way of Cairns-type intermediates. Since predominantly early intermediates were analyzed in this study, it was not possible to determine whether fork movement takes place in one or both directions from the origin following initiation.

Once the BPV genome is established as a plasmid in a transformed cell line, its copy number is maintained at a constant level. Hence, there must be some mechanism that ensures that there is an exact, or nearly exact, doubling of BPV DNA during each cell cycle. There are several different ways in which a constant average copy number could be maintained. One possibility is that viral DNA replication could be limited by the availability of some required factor. Viral genomes would replicate at random until this factor was exhausted, and then replication would cease until the factor was replenished during the next cell cycle. A prediction of this model is that some DNA molecules would replicate more than once during a cell cycle, and a similar fraction would not replicate at all. An alternative model is that each viral genome replicates once and only once per cell cycle. A requirement of such a model is that some mechanism exists that distinguishes between those viral genomes that have undergone a round of DNA replication and genomes that have yet to be replicated. The mode of replication of BPV DNA has been analyzed by means of density labeling experiments (342). Mouse cells trans-

formed by BPV and containing an average of ~150 viral genomes per cell were labeled with bromodeoxyuridine during exponential growth. At intervals, plasmid DNA was isolated and analyzed by density gradient centrifugation. The results of this experiment clearly showed that during one cell cycle, nearly all of the viral DNA shifted to the hybrid density expected of molecules that had undergone a single round of semiconservative DNA synthesis; essentially no viral DNA with the density expected for molecules that had undergone more than one round of replication was observed until the cells were labeled for a period longer than a single cell division cycle. The simplest interpretation of this experiment is that the overwhelming majority of viral DNA molecules replicate once and only once per cycle. Very different results, however, were obtained in a variation of this experiment in which the density-labeled cells that had completed S-phase were selected by mitotic shake-off (343). In this experiment, up to 20% of the viral DNA labeled during a single S-phase banded at the density indicative of molecules that had undergone multiple rounds of DNA replication. The reason for the discrepancy between these results is not clear. It is possible that certain cell culture conditions may promote the transient appearance of cells in which viral DNA replication becomes unregulated. A small percentage of such cells could potentially result in the appearance of a relatively high proportion of viral DNA molecules that had undergone multiple rounds of DNA synthesis. Whatever the explanation for the latter results, it seems clear that under at least some conditions, BPV DNA replicates once and only once per cell cycle. Thus, in this respect, BPV DNA replication appears similar to chromosomal DNA replication.

Genetic Analysis of DNA Replication

CIS-ACTING ELEMENTS Two distinct *cis*-acting sequences in the BPV genome have been found to allow autonomous replication of plasmid DNAs in the presence of BPV gene products. These elements were first identified by cloning fragments of BPV into a vector expressing the gene encoding neomycin resistance (333). The cloned DNAs were then introduced into mouse cells transformed by BPV, neomycin-resistant colonies were selected, and the physical state of the marker gene was analyzed. In most cases, the plasmid DNA was found to be integrated into the chromosomal DNA. Two small segments of BPV DNA (plasmid maintenance sequences; PMS) gave rise to neomycin-resistant colonies in which the marker gene was maintained as an extrachromosomal nuclear plasmid. When these same plasmids were introduced into untransformed mouse cells (not containing any additional BPV sequences), they failed to replicate extrachromosomally; hence, autonomous replication also requires *trans*-acting BPV gene products (see below).

One of the plasmid maintenance sequences, PMS-1, was mapped to a

521-bp segment (nucleotides 6945–7476) within the LCR. The other, PMS-2, was mapped to a 140-bp region (nucleotides 1515–1655) within the E1 ORF. The function of these sequences is not known with certainty. PMS-1 is located very near to the position at which the origin of replication of BPV was mapped (336). It seems likely, therefore, that PMS-1 is the site at which DNA replication is initiated. The sequences of PMS-1 and PMS-2 contain a region of extensive homology (333). Therefore, it seems reasonable to assume that PMS-2 may also function as an origin of replication. In the electron microscopic analysis of replication intermediates described earlier, no molecules with replication eyes centered on PMS-2 were observed. It is possible that initiation of replication at PMS-2 is much less efficient and, in the presence of PMS-1, does not occur at a measurable frequency. An analysis of replicative intermediates of plasmids that contain PMS-2 but lack PMS-1 has not been reported.

There is evidence to suggest that the plasmid maintenance sequences may serve other functions in addition to a role in the initiation of replication. Plasmids containing either PMS are stably maintained in cells at a constant copy number in the absence of selection. This observation implies that the PMS elements may contribute to the controlled partitioning of plasmids during cell division (333). In addition, the PMS may function as part of a system that suppresses integration of plasmid DNA. As mentioned, when recombinant plasmids containing PMS-1 or PMS-2 linked to a selectable marker are introduced into BPV-transformed cells, the marker gene is invariably found as an extrachromosomal plasmid. When the same recombinants are used to transfect untransformed cells, the marker gene is always integrated. Moreover, if a plasmid containing PMS-1 or PMS-2 is introduced into transformed cells together with an unlinked selectable marker gene, the marker gene is integrated, while the plasmid containing the PMS is again found as an extrachromosomal plasmid (333).

The sequences necessary for the function of PMS-1 have been analyzed in detail by looking at the effect of various mutations in PMS-1 on the ability of plasmids to replicate transiently following transfection into BPV-transformed cells (347). This transient replication system very likely corresponds to the amplification of viral DNA that must occur during the establishment of a stable final copy number of ~200 from a single infecting genome. Two distinct domains of PMS-1 have been identified by this means. Domain 2 (nucleotides 7116–7224) completely overlaps the region of homology with PMS-2. Domain 1 (nucleotides 6707–6848) appears to be a transcriptional enhancer: the function of domain 1 is independent of its orientation and exact distance away from domain 2, and can be replaced by known enhancer elements of other viruses. Whether the function of domain 1 in DNA replication relates in any way to transcriptional enhancer function is not known. In this context, however, it is also of interest that domain 2 also contains

transcriptional regulatory signals. An mRNA start site (called P1) has been located within domain 2 at nucleotide 7186 (344). Deletion analysis has revealed that the controlling elements required for transcription initiation at this site are located downstream of the site of initiation of transcription, also within the boundaries of domain 2. Deletion of 23 base pairs between nucleotides 7187 and 7234 was shown to abolish both PMS-1-dependent DNA replication and transcription from P1. On the basis of these results it has been suggested that a cellular transcription factor(s) required for transcription from P1 may also play a key role in the initiation of DNA replication. According to this model, the function of the domain 1 enhancer element is to potentiate the binding of the appropriate transcription/replication factors to domain 2. It is not known whether an enhancer element is also required for stable plasmid replication. It is also unclear whether PMS-2 will support transient replication, and if so, whether such replication also depends on the presence of an enhancer element. There is no known transcriptional start site in the vicinity of PMS-2. Therefore, it seems unlikely that there is an obligatory requirement for transcription per se in PMS function, although there is no evidence to rule out the possibility that transcription from PMS-2 takes place on plasmids that lack PMS-1. An evaluation of the role that transcription factors and/or transcription play in the various aspects of BPV DNA replication will almost certainly depend on the development and biochemical analysis of a PMS-dependent in vitro replication system.

TRANS-ACTING VIRAL GENE PRODUCTS The BPV genes involved in DNA replication have also been investigated using both transient assays and assays involving the establishment of stable extrachromosomal plasmids. Deletion mutants lacking ORFs E2, E3, E4, and E5 were shown to be capable of autonomous replication (333). Mutations in other ORFs have now defined three different complementation groups of mutants that are defective in autonomous replication, and two additional groups of mutants in which the control of plasmid copy number is altered (337, 345–347).

Mutants with lesions in the 3' portion of the E1 ORF transform cells with the same efficiency as wild-type DNA, but the mutant DNA is invariably integrated into the chromosomal DNA of the transformed cell rather than replicating as an autonomous plasmid (345). These mutants (called rep^- or R^-) also fail to replicate transiently following transfection, and cells transformed by R^- mutants do not support the transient replication of plasmids containing PMS elements. Autonomous replication by R^- mutants can be complemented in both transient assays and stable transformation assays by mutants in other complementation groups. Thus, the 3' portion of the E1 ORF encodes a protein that may be directly involved in BPV DNA replication. The product(s) of the R gene has not yet been identified.

Mutants with lesions in the 5' portion of the E1 ORF define a second complementation group (referred to as modulator$^-$ or M$^-$) that are defective in autonomous replication (348, 349). These mutants transform cells with very much lower efficiency than wild-type DNA. As in the case of R$^-$ mutants, the mutant DNA is always integrated into the chromosomal DNA of transformed cells. Unlike R$^-$ mutants, however, these mutants replicate as well as wild-type BPV DNA in transient assays. M$^-$ mutants therefore define a function that is required for plasmid maintainance but not DNA synthesis per se. There is additional genetic evidence (see below) that the M function is a repressor that is required to prevent "runaway" replication of the BPV replicon. The available evidence strongly suggests that even though the M function and the R function are both encoded in the same open reading frame (E1), these two functions are the products of two distinct genes (348). First, the two groups of mutants complement each other fully. When R$^-$ mutants are cotransfected with M$^-$ mutants, both mutant genomes replicate in transient assays, and stably transformed lines derived from such transfections contain both genomes as extrachromosomal plasmids. Second, frame shift mutations in the M gene, at the 5' end of the E1 ORF, do not affect R function. Finally, the product of the M gene has been identified in BPV-transformed cells using antisera prepared against the amino-terminal portion of the E1 ORF expressed in *E. coli* (340); the apparent size of the M protein on SDS gels is 23 kd, significantly smaller than the predicted size of the complete E1 ORF (68 kd). Spliced mRNAs that could account for the synthesis of an amino-terminal truncation of the E1 ORF have been identified.

Recently, a third complementation group of mutants with properties intermediate between those of R$^-$ and M$^-$ mutants has been identified (M. Lusky, personal communication). These mutants have lesions in the E8 ORF, which is completely embedded within the E1 ORF in a different translational reading frame. Missense mutations in the E8 ORF that leave the E1 ORF unaltered have been constructed by in vitro mutagenesis. These mutants transform cells at the same efficiency as wild-type DNA. As in the case of both R$^-$ and M$^-$ mutants, the mutant DNA is not maintained as an extrachromosomal plasmid. These mutants therefore define another viral product that is essential for plasmid maintainance. E8 mutants are able to replicate DNA in transient assays, although at a reduced rate compared to wild-type or M$^-$ mutants. E8 mutants complement R$^-$ mutants in transient assays and both R$^-$ and M$^-$ mutants in assays for stable autonomous replication. The protein responsible for the function lacking in these mutants has not yet been identified.

Mutations in the E6 and E7 ORFs do not affect the ability of BPV to replicate as an autonomous plasmid, but can result in a nearly 100-fold reduction in plasmid copy number, from about 200 to 1–5 genomes per cell (345). Analysis of cDNA clones has revealed two classes of mRNAs that

contain sequences from this region. One class contains the E6 ORF intact, and the other class contains a splice that joins a portion of the E6 ORF to a portion of the E7 ORF to generate a putative E6/7 fusion protein. Mutants with lesions that specifically affect either the E6 product or the E6/7 product can complement each other and so define two distinct genes; however, both groups that display this altered copy number phenotype are collectively referred to as cop$^-$ mutants (345, 347, 348). When cells are transfected with cop$^-$ mutants, the mutant genomes initially replicate to high copy number, but with continued passage of the transformed cells the copy number gradually declines to 1–5 copies per cell (347). This copy number is then maintained stably for many generations. The mechanism by which the E6 and E6/7 genes exert an influence on copy number is not known. Analysis of many individual subclones derived from a single transformed cell has shown that this low average copy number of cop$^-$ mutants is not due to a gross defect in segregation. It has been reported that the E6 and/or the E6/7 genes influence the activity of the enhancer element in PMS-1 (L. Turk, quoted in Ref. 347). Since the promoter for the M and R genes lies within PMS-1, it is possible that the cop genes affect the level of expression of the other genes that are involved in autonomous DNA replication. Alternatively, the level of expression from the promoter within PMS-1 may directly affect the initiation of DNA replication.

Complementation tests with E6 and E6/7 mutants have shed some light on the functions of some of these genes. When E6 or E6/7 mutants are cotransfected with wild-type BPV DNA or with mutants in other complementation groups, both DNAs replicate transiently and become established as high-copy-number plasmids (345, 347, 348). Hence, the E6 and E6/7 gene products function in *trans*. On the other hand, if cells are first transformed with a cop$^-$ mutant and then supertransfected with wild-type DNA, the wild-type DNA is not amplified transiently and becomes established at low copy number (349). This result suggests that wild-type BPV encodes a function that represses plasmid amplification, and the cop gene products play some role in controlling the level of this repressor once stable plasmid copy number has been established. The available evidence suggests that the M gene encodes this repressor. When an M$^-$ mutant is used to supertransfect cells carrying a low-copy-number mutant, the incoming M-DNA is transiently amplified but the resident low-copy mutant genome is not (347). The simplest explanation for this result is that the product of the M gene represses plasmid amplification, but in the steady state the protein is sequestered in some fashion so that it cannot readily work in *trans* on newly introduced DNA. In this context, it is also of interest to note that when cotransfected into cells along with a selectable marker, M$^-$ mutants decrease the efficiency of transformation nearly 100-fold. This low transformation efficiency may well be due to a

lethal effect of unconstrained replication of the M^- replicon, which lacks a critical negative element of the copy number regulatory system.

It is clear that BPV encodes a regulatory system that acts to repress amplification of viral DNA when plasmid copy number reaches a certain critical level (100–200 copies per cell). There is also evidence that the BPV repression mechanism can function with a heterologous replication origin (350–352). A hybrid replicon was constructed containing the 5.4-kb BPV-transforming fragment and the SV40 origin of replication. This chimeric molecule was introduced into cells expressing the SV40 T-antigen. Transient amplification of this hybrid plasmid was suppressed relative to a control plasmid containing the SV40 origin but lacking the BPV sequences, even when both plasmids were introduced simultaneously into the same cells. In addition, it was possible to establish stable cell lines in which the hybrid plasmid was maintained at a constant copy number. Density transfer experiments showed that as in the case of latent BPV genomes, once the steady state copy number of the hybrid plasmid was reached, every plasmid replicated once and only once per cell division cycle. Two BPV elements were required in *cis* for the suppressive effect on replication. One element coincided with PMS-1 and the other was near PMS-2. At least one BPV gene product is required in *trans*. Deletions removing the 5' portion of the E1 gene abolished the suppressive effect on replication, and supressed replication could be restored by cotransfection with wild-type BPV DNA. It therefore seems likely that the M gene is part of a BPV regulatory system that can repress DNA replication from a heterologous origin when it is linked to specific BPV *cis*-acting sequences. It will be of considerable interest to elucidate the mechanisms by which this regulatory system interacts with the cellular replication machinery.

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Abundant Expression of Herpes Simplex Virus Glycoprotein gB Using an Adenovirus Vector

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Herpes simplex virus type 1 (HSV-1) glycoprotein B (gB) is a major component of infected cell membranes and virion envelopes. Glycoprotein B is known to be essential for entry of viruses into cells and may play important roles in virus-induced cell fusion and other alterations in cell morphology. In order to study the biochemical and immunological properties of gB in isolation from other HSV-1 polypeptides we have constructed human adenovirus vectors capable of expressing high levels of gB. The gB gene was coupled to the SV40 early promoter and inserted into the E3 region of two adenovirus vectors, one in which the E1 region was deleted (AdgB-1) and another which contained E1 sequences (AdgB-2). In AdgB-1 the orientation of the chimeric gB-SV40 gene was right to left, i.e., opposite to the direction of late and E3 mRNA transcription, whereas in AdgB-2 the orientation was left to right. Human 293 cells which express E1 functions supported replication of AdgB-1 and gB was expressed in these cells but not in mouse cells and only at very low levels in human cells other than 293. Replication of AdgB-2 was not limited to 293 cells and the virus was able to induce synthesis of gB at levels equal to or higher than those expressed in HSV-1-infected human or mouse cells. Microscopic examination of AdgB-2-infected cells revealed extensive vacuolization in a manner completely uncharacteristic of adenovirus-infected cells, and fluorescent antibody staining indicated that gB was not only present at the cell surface but also concentrated in the cytoplasmic vacuoles. © 1988 Academic Press, Inc.

INTRODUCTION

Glycoprotein B (gB) is one of a number of membrane proteins specified by herpes simplex virus type 1 (HSV-1) which are present in host membranes and in the virion envelope (reviewed in Spear, 1984). There is good evidence that gB is an essential component of the virion envelope, being involved in membrane fusion events required for virus penetration into cells (Bzik *et al.*, 1984; Cai *et al.*, 1987; Little and Schaffer, 1981; Sarmiento *et al.*, 1979). It is also likely that gB is involved in changes in the social behavior of infected cells since HSV-infected cells round up, clump together, and, depending on the strain of virus and cell type, fuse with one another or form syncytia (Hoggan and Roizman, 1959; Manservigi *et al.*, 1977; Spear 1984), and certain syncytial (syn) mutations have been mapped to the gene encoding gB (Bizik *et al.*, 1984; DeLuca *et al.*, 1982; Sarmiento *et al.*, 1979). In addition, although viruses with mutations in the gB gene can bind to cells, evidence has been presented that gB may play a role in virus attachment to cells (Johnson *et al.*, 1984). The essential role or roles carried out by gB may also be important in the replicative cycles of other herpesviruses because gB homologs have been described for a number of human (Gong *et al.*, 1987; Pellet *et al.*, 1985a; Edson *et al.*, 1985; Emini *et al.*, 1987) and animal herpesviruses (Snowden *et al.*, 1985).

There is good evidence that gB, as well as other viral glycoproteins, plays an important role in the host immune response. Neutralizing antibodies are produced against gB in infected animals and polyclonal antisera to gB mediate immune cytolysis of HSV-infected cells (reviewed in Spear, 1984). In addition, purified gB can stimulate human memory T lymphocytes (Torseth *et al.*, 1987; Zarling *et al.*, 1986) and helper T cells induced by purified gB can protect mice against HSV-1 infection (Chan *et al.*, 1985). However, there is also evidence that gB is not a major target for cytotoxic T lymphocytes in mice (Glorioso *et al.*, 1985; Rosenthal *et al.*, 1987).

A number of different cell lines expressing HSV-1 gB have been described (Arsenakis *et al.*, 1986; Cai *et al.*, 1987; Pachl *et al.*, 1987; Rosenthal *et al.*, 1987). Cells constitutively expressing gB were able to complement viruses carrying mutations in the gB gene (Arsenakis *et al.*, 1986; Cai *et al.*, 1987) and a secreted form of gB was purified from medium conditioned by hamster ovary cells transfected with a truncated form of the gB gene (Pachl *et al.*, 1987). However, it is our experience that the expression of gB in mouse and human cell transformants is much lower than in HSV-infected cells (Rosenthal *et al.*, 1987; D. C. Johnson, unpub-

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lished results). Adenovirus vectors have proven useful for achieving high-level expression of a variety of foreign genes and have the advantage over transformed cell lines that one can obtain expression under a variety of different conditions by varying such parameters as the host cell or the multiplicity of infection. Viral vectors have the additional advantage of allowing for expression of foreign antigens *in vivo* to study immunological effects and may offer the possibility of development of novel vaccines. We have constructed adenovirus 5 (Ad5)-based vectors containing and expressing gB coding sequences for use in studies on the effects of high-level expression of gB in various host cells in the absence of other HSV gene products and for *in vivo* studies on the immunological properties of adenovirus expression vectors.

MATERIALS AND METHODS

Cells and viruses

Wild-type Ad5, Sub54, and AdgB-2 were grown in HeLa or KB cells and titered on 293 cells as described (Graham *et al.*, 1978). AdgB-1 was grown and titered on 293 cells (Graham *et al.*, 1977) which were maintained in Joklik's modified medium supplemented with 10% horse serum. The human osteosarcoma cell line R970-5 (Rhim *et al.*, 1975) (obtained from K. Huebner and C. Croce, Wistar Institute, and here referred to as R970 cells) and Vero cells were grown in minimum essential medium-α (α-MEM) supplemented with 7% fetal calf serum. Mouse LTA cells (Graham *et al.*, 1980; Kit *et al.*, 1963) and Z4 cells (Persson *et al.*, 1985) were grown in α-MEM supplemented with 10% fetal calf serum. HSV strain F (obtained from P. G. Spear, University of Chicago) was propagated and titered on Vero cell monolayers.

Construction of plasmids

Restriction endonucleases and other DNA modifying enzymes were purchased from Bethesda Research Laboratories (Burlington, Ontario) or Boehringer-Mannheim, Inc. (Dorval, Quebec), and were used according to the vendor's recommendations. *Xba*I linkers were purchased from Pharmacia Ltd. (Dorval, Quebec). All plasmids were constructed by standard protocols (Maniatis *et al.*, 1982) except for plasmids containing circularized adenovirus DNA which are generated according to methods described elsewhere (Ghosh-Choudhury *et al.*, 1987; Graham, 1984; Ruben *et al.*, 1983). Bacterial strains (HB101, LE392, and HMS174) were made competent for transformation by $CaCl_2$ treatment (Mandel and Higa, 1970). Plasmid DNA was prepared by the alkaline lysis method of Birnboim and Doly (Birnboim and Doly, 1979) for both small-scale preparations and large-scale purification. Large-scale preparations were purified further by CsCl–ethidium bromide density gradient centrifugation.

Transfection of 293 cells and screening of recombinant viruses

Subconfluent monolayers of 293 cells were transfected or cotransfected with appropriate plasmids (as shown in Figs. 1 and 2) using the calcium technique (Graham and van der Eb, 1973). After 8–10 days plaques were picked and grown in 293 cells, and virus DNA was analyzed by restriction enzyme digestion and agarose gel electrophoresis to identify recombinant vectors. Candidate plaque isolates were twice plaque purified and reanalyzed prior to preparation and use of large-scale virus stocks.

Radiolabeling and preparation of cellular extracts

HeLa, 293, R970, LTA, and Z4 cells growing in 35- or 60-mm dishes were infected with AdgB-1, AdgB-2, or HSV-1 using 5, 10, 50, 100, or 500 PFU per cell of a given virus as indicated in the figure legends; after 2 hr of adsorption the virus innoculum was removed and α-MEM + 2% fetal calf serum was added. Cells infected with HSV-1 were labeled from 4 to 12 hr postinfection and those infected with AdgB-1 or AdgB-2 were labeled from 20 to 36 hr postinfection; in each case the cells were washed twice with medium lacking methionine and then labeled using medium 199 lacking methionine and containing 25 μCi/ml [^{35}S]methionine. HSV-, AdgB-1-, and AdgB-2-infected cells were pulse-labeled at various times after infection by first washing the monolayers three times with medium 199 lacking methionine and incubating the cells with [^{35}S]methionine (100 μCi/ml) for 10 min in medium 199 lacking methionine. The cells were immediately extracted with 2 ml (35-mm dishes) or 5-ml (60-mm dishes) 50 m*M* Tris–hydrochloride (pH 7.5)–100 m*M* NaCl–1% Nonidet-P40 (NP-40)–0.5% sodium deoxycholate (DOC)–1 mg of bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO)/ml–0.1 mg of aprotinin (Sigma)/ml–0.5 m*M* phenylmethylsulfonyl fluoride (PMSF, Sigma)/ml (NP-40–DOC extraction buffer). Extracts were frozen at −20° until immunoprecipitations were performed.

Pulse–chase experiments and trypsin treatment of cell surfaces

R970 cells grown in 35-mm dishes were labeled for 10 min with [^{35}S]methionine (100 μCi/ml) or labeled for 10 min and incubated with medium containing excess

unlabeled methionine for various times and then immediately extracted with NP-40–DOC extraction buffer. In other experiments the monolayers were washed twice with phosphate-buffered saline (PBS: 10 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, 140 m*M* NaCl, 3 m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, pH 7.4) following the pulse or chase period and then incubated with 1.5 ml of PBS or 1.5 ml PBS containing trypsin (0.5 mg/ml: type XIII, Sigma) for 10 min at 37° PMSF (7.5 μl; 10 mg/ml in EtOH) was added immediately and then the cells were washed twice with 50 m*M* Tris–hydrochloride (pH 7.5), 100 m*M* NaCl containing soybean trypsin inhibitor (2.5 mg/ml; Sigma), BSA (5 mg/ml; Sigma), aprotinin (0.2 mg/ml; Sigma), and 1.0 m*M* PMSF at 4°. The cells were extracted in NP-40–DOC extraction buffer and extracts frozen at −20°.

Immunoprecipitation and gel electrophoresis

Immunoprecipitations of gB synthesized in HSV-1-, AdgB-1-, and AdgB-2-infected cells were performed as described previously (Johnson and Feenstra, 1987). Cell extracts were sonicated, clarified by centrifugation, and incubated for 1–2 hr at 4° with rabbit anti-gB serum No. 67 (Johnson and Spear, 1982) provided by P.G. Spear (University of Chicago) or with mouse monoclonal antibody 15βB2 provided by Dr. S. Bacchetti (McMaster University), which precipitates HSV-1 and HSV-2 gB. Protein A–Sepharose beads (Pharmacia) were added and the mixture was incubated for 1 to 2 hr at 4° on a rotating wheel after which the beads were washed three times with RIPA buffer: 50 m*M* Tris–hydrochloride (pH 7.2), 150 m*M* NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 0.5% DOC; and precipitated proteins were eluted with two-fold-concentrated sample buffer (100 m*M* Tris–hydrochloride, pH 8.3, 4% SDS, 4% α-mercaptoethanol, 20% glycerol, bromphenol blue). Samples were electrophoresed in 8.5% *N,N*-diallyltartardiamide (DATD) crosslinked polyacrylamide gels as described by Heine *et al.* (1974) at 60 V for 16 hr. Gels were infused with 2, 5-diphenyloxazole (PPO) by the procedure of Bonner and Laskey (1974) and then dried and placed in contact with Kodak XAR film.

S1 and primer extension analysis

Cytoplasmic RNA was prepared from HSV-1 infected R970 cells (7 hr postinfection with 10 PFU/cell) and from AdgB2-infected R970 cells (36 hr postinfection, 20 PFU/cell) according to Berk and Sharp (1977). In each hybridization reaction, 20 μg cytoplasmic RNA was used. The probe used for S1 analysis was a 189-nt double-stranded fragment, extending from an *Rsa*I site within the gB coding sequences to an *Nco*I site upstream of the SV40 TATA box, 5′-end-labeled at the *Rsa*I site (see Fig. 6). Hybridization under R-looping conditions (80% formamide, 56°) and S1 digestion were carried out as described (Smiley *et al.*, 1987).

Primer extension experiments used a 5′-end-labeled synthetic primer (5′-ACGTGAGATATACGCCGGGCGGGTA-3′) purchased from the Central Facility of the Institute of Molecular Biology and Biotechnology, McMaster University. The 5′-end of this primer corresponds precisely to the 5′-end of the DNA fragment used for S1 analysis. Primer extension using AMV reverse transcriptase was as previously described (Smiley *et al.*, 1987). S1 digestion products and primer extension products were fractionated on 8% DNA sequencing gels.

Light microscopy and immunofluorescence

R970 cells grown on glass coverslips in 35-mm dishes were infected for various times with HSV-1 (5 PFU/cell), AdgB-2 (20 PFU/cell), or Sub54 (20 PFU/cell), fixed with methanol, stained with Wright Giemsa stain, and mounted in 70% glycerol: 30% PBS on glass microscope slides. The slides were viewed and photographed using bright field optics with a Zeiss microscope. Immunofluorescence experiments were performed with cells grown on glass coverslips, infected as above, and fixed with 3.7% formaldehyde in PBS for 30 min at 20°. The cell monolayers were washed twice with PBS, incubated for 20 min with 50 m*M* NH_4Cl in PBS, and then in some cases permeabilized with 0.1% Triton X-100 in PBS for 10 min at 22°. The cells were washed twice with PBS containing 0.2% gelatin and 0.3% BSA (PBS/gelatin/BSA), incubated in PBS/gelatin/BSA for 1–16 hr, and then incubated with 100 μl of PBS/gelatin/BSA containing 5 μl of 15βB2 ascites fluid for 1 hr at 22°. The coverslips were washed three times with PBS/gelatin/BSA, incubated with affinity-purified, fluoresceinated rabbit anti-mouse IgG (Cappel Laboratories, Cochranville, PA) for 1 hr at 22°, and then washed three times with PBS. The coverslips were mounted in 50% (v/v) glycerol–50% (v/v) PBS containing *p*-phenylenediamine (10 μ/ml) and viewed and photographed using a Zeiss fluorescence microscope.

RESULTS

Construction of AdgB-1 and expression of gB in AdgB-1-infected cells

An adenovirus containing the HSV-1 gB gene under the control of the SV40 early promoter and lacking E1 sequences was constructed as described in Fig. 1. The plasmid pSV2gB containing gB structural se-

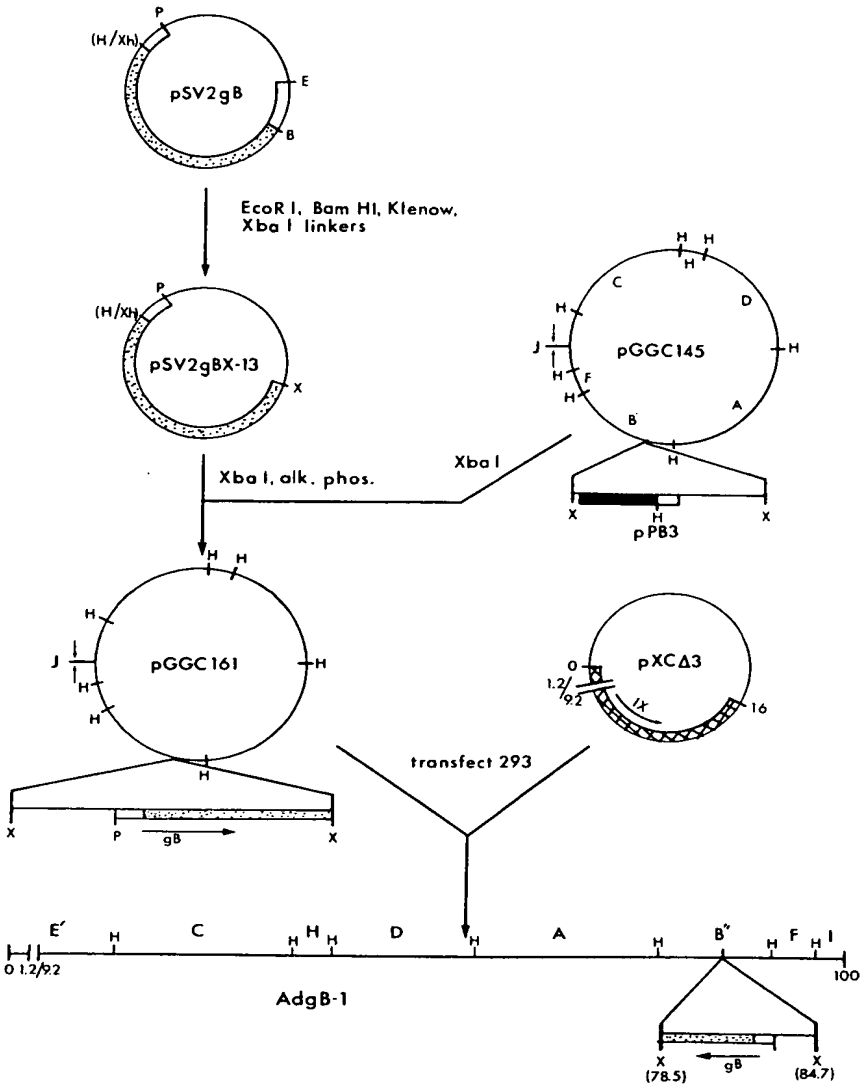


FIG. 1. Outline of the construction of AdgB-1. Plasmid pSV2gB (Rosenthal *et al.*, 1987) was digested with *Eco*RI and *Bam*HI, treated with the Klenow fragment of DNA polymerase I, and ligated to *Xba*I linkers. The resulting plasmid, pSV2gBX-13, was digested with *Xba*I, treated with calf intestinal alkaline phosphatase and ligated to *Xba*I-digested pGGC145. Plasmid pGGC145 is infectious when transfected onto 293 cells and contains Ad5 *d*/E1,3 sequences and an insert of pPB3, a plasmid containing the neomycin-resistance gene and the λ cos packaging sequences (Ghosh-Choudhury *et al.*, 1987). The resulting plasmid, pGGC161, contains the pSV2gBX-13 sequences inserted at the single *Xba*I site of pGGC145 in place of pPB3. Plasmid pGGC161 was cotransfected with pXCΔ3 (containing the left 16% of Ad5 and a deletion of E1 sequences between a *Pvu*II site at 450 bp and a *Bgl*II site at 3328 bp) into human 293 cells in 60-mm dishes. Plaques were picked and expanded and viral DNA was analyzed by restriction enzyme digestion. The *Hin*dIII fragments of AdgB-1 are shown below. Fragments A, C, D, F, H, and I are identical to wild-type Ad5 *Hin*dIII fragments, B″ is 10.0 kb in size and includes pSV2gBX-13 sequences inserted at the unique *Xba*I site, and E′ is 3.2 kb. Stippled boxes represent HSV-1 gB sequences, open boxes represent SV40 sequences, the closed box in pGGC145 represents the coding sequences for the neomycin resistance gene and the hatched box in pXCΔ3 represents the left 16% of Ad5 with a deletion in E1. Restriction sites: *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Pvu*II (P), *Xba*I (X), *Xho*I (Xh), the *Hin*dIII/*Xho*I junction (H/Xh), and the junction of Ad5 ends (J) are indicated. Numerals indicate locations in the wild-type Ad5 genome in map units.

quences fused to the SV40 early promoter has been described previously (Rosenthal *et al.*, 1987). A *Xba*I site was generated in pSV2gB by collapsing the *Eco*RI and *Bam*HI sites around an *Xba*I linker and the resulting plasmid, pSV2gBX-13, was inserted in its entirety in place of pPB3 sequences in the infectious plasmid pGGC145 (Ghosh-Choudhury *et al.*, 1986). The resulting plasmid, pGGC161, contained Ad5 *d*/E1,3 (Haj-

Ahmad and Graham, 1986) sequences with deletions in the E1 region (1.0–10.6 map units) and E3 region (78.5–84.7 map units) and an insertion of pSV2gBX-13 (6.0 b) at the *Xba*I site in E3. Because Ad5 *dl*E1,3 carries extensive deletions totalling 5349 bp we expected that inserts as large as 7–7.5 b could be inserted and viruses able to replicate on 293 cells could be isolated. However, repeated attempts to produce infectious virus by transfection of 293 cells with pGGC161 failed. We subsequently discovered that by restoring the coding sequences for protein IX the packaging capacity of our adenovirus vectors could be increased and infectious recombinants could be recovered (Ghosh-Choudhury *et al.*, 1987). We therefore cotransfected 293 cells with pGGC161 and pXCΔ3, which contains the left 16% of Ad5 and a deletion of E1 sequences which leaves the protein IX gene intact (see Fig. 1). Virus plaques (three to four per 60-mm dish) appeared after 6 to 8 days and several were picked and viral DNA from these was analyzed by restriction enzyme digestion. All of the virus isolates contained the DNA structure outlined in Fig. 1 and one virus, AdgB-1, was used in further experiments.

LTA, HeLa, and 293 cell monolayers were infected with AdgB-1 at various multiplicities of infection (m.o.i.). After 24 hr 293 cells infected with 10, 100, or 500 PFU/cell showed signs of infection whereas the other cells were normal in appearance. AdgB-1-infected cells were labeled with [^{35}S]methionine 8 hr after infection, cell extracts were prepared at 24 hr, and gB was immunoprecipitated with a rabbit polyclonal anti-gB serum. In 293 cells we detected gB as well as the immature form of the glycoprotein, pgB, and found that the synthesis of the glycoprotein was greatest at high multiplicities of infection (Fig. 2). However, we were unable to detect synthesis of gB in AdgB-1-infected LTA or HeLa cells at these relatively early times after infection (Fig. 2). It appeared that the virus did not replicate well in these cells and even after 60 hr the cells showed little sign of virus cytopathic effect. This result was not unexpected because the virus lacked the E1 region of adenovirus which is essential for replication in cells other than 293.

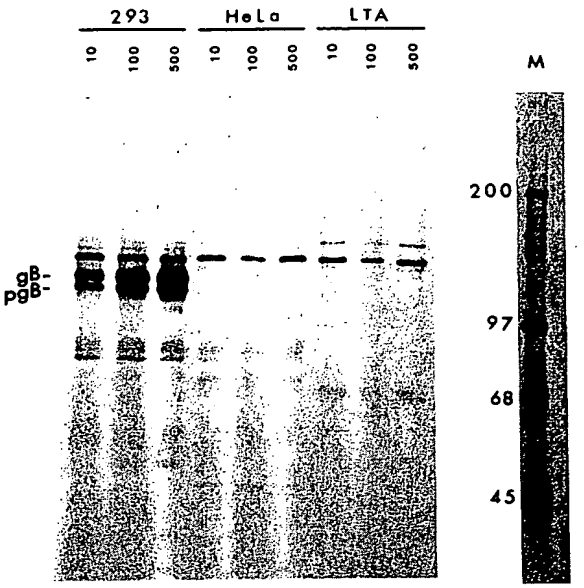


FIG. 2. Expression of gB in AdgB-1-infected cells. HeLa, 293, and LTA cells grown in 60-mm dishes were infected with AdgB-1 using 10, 100, or 500 PFU per cell and then labeled with [^{35}S]methionine at 8 hr postinfection. Cell extracts were made at 24 hr. postinfection and gB was immunoprecipitated with rabbit polyclonal anti-gB serum No. 67 (Johnson and Spear, 1982). The precipitated proteins were eluted and electrophoresed in 8.5% DATD-crosslinked polyacrylamide gels which were infused with PPO, dried, and placed in contact with Kodak XAR film. The right side of the figure shows protein molecular weight markers of 200, 97, 68, and 45 kDa. The left side indicates the positions of gB and the immature form of gB, pgB.

Construction of AdgB-2 and expression of gB in human and mouse cells

In order to isolate an adenovirus vector able to replicate and direct the synthesis of gB in cells other than 293 cells we constructed a vector containing both pSV2gB and E1 sequences. Since this necessitated a reduction in the size of inserted DNA, a second *Xba*I site was engineered at the unique *Nde*I site of pSV2gBX13 so that the SV40 promoter and gB structural sequences could be excised from the plasmid with *Xba*I and inserted into the *Xba*I site of pFGdX1 which contains the right 40% of adenovirus type 5 (Ad5) and a deletion of E3 sequences (Haj-Ahmad and Graham, 1986). The resulting plasmid pgBdX17 was cotransfected with pFG154neo, which contains the entire adenovirus genome but is not infectious, presumably because the pPB3 insert at the *Eco*RI site (76 m.u.) disrupts the protein VIII gene. Recombination between Ad5 sequences in pgBdX17 and pFG154neo in the region of 59.5–76.0 map units was therefore expected to yield an infectious virus containing the SV2gB sequences. Approximately 8 days after transfection a single plaque appeared on 293 monolayers. This plaque displayed a markedly different morphology from that of wild-type Ad5 virus with highly vacuolated cells bordering the plaque. Viral DNA from this isolate was analyzed using restriction enzymes and found to have the structure shown in Fig. 3. The resulting virus, designated AdgB-2, was plaque purified and propagated in HeLa and KB cells for further study.

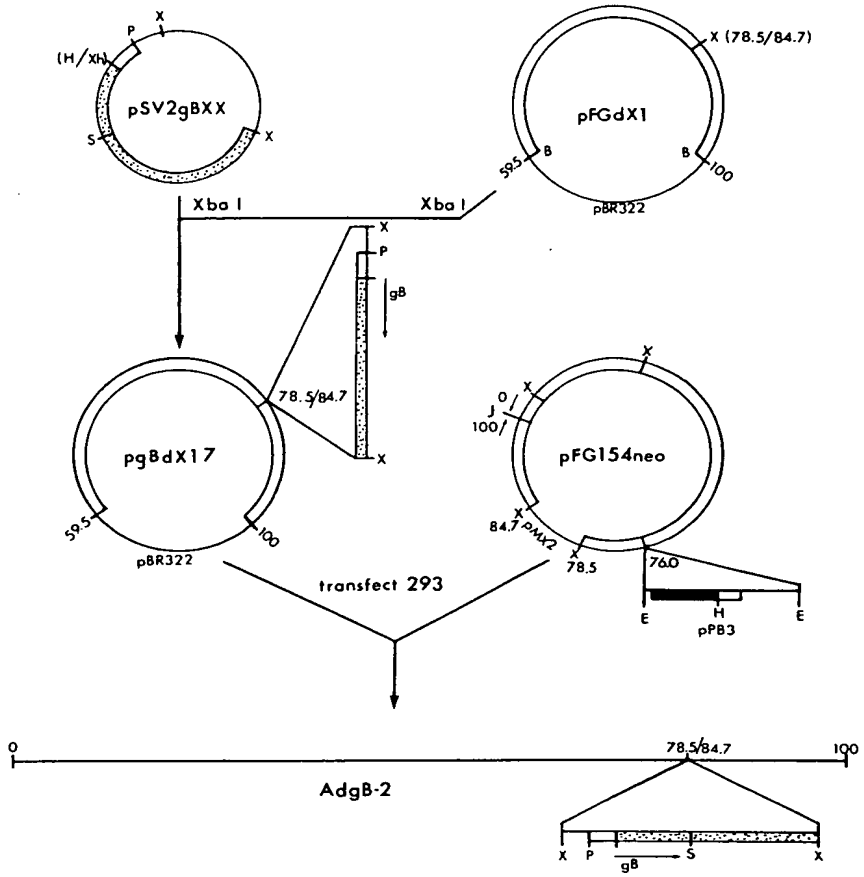


FIG. 3. Construction of AdgB-2. Plasmid pSV2gBXX was constructed from pSV2gX-13 (Fig. 1) by digesting with *Nde*I, treating the DNA with the Klenow fragment of DNA polymerase I and ligating *Xba*I linkers into the gapped DNA. The 4.1-kb *Xba*I fragment of pSV2gBXX was purified from an agarose gel and ligated to *Xba*I-digested pFGdX1 which contains the right 40% of Ad5 *dl*E1,3 (Haj-Ahmad and Graham, 1986). The resulting plasmid was named pgBdX17. Plasmid pFG154neo contains an insert of pMX2 at the *Xba*I site (78.5/84.7 m.u.) of Ad5 *dl*E3, an insert of pPB3 sequences at the *Eco*RI site (76.0 m.u.) of Ad5 *dl*E3, and has Ad2 sequences extending from the left end of the virus to a position at 41–50 m.u. The derivation of this plasmid will be described in more detail elsewhere. 293 cells were cotransfected with pFG154neo and pgBdX17 and after 8 days a single plaque appeared. The plaque was isolated, a virus stock was grown on HeLa cells, and viral DNA was analyzed by restriction enzyme digestion. Restriction sites *Hin*dIII (H), *Eco*RI (E), *Pvu*II (P), *Sal*I (S), *Xba*I (X), *Xho*I (Xh) are shown where relevant. J represents the junction of Ad2 (left end) and Ad5 (right end) termini, stippled boxes indicate HSV-1 gB DNA, and open boxes indicate adenovirus DNA in plasmids pFGdX1, pFG154neo, and pgBdX17 or the SV40 promoter in pSV2gBXX and pPB3.

HeLa and 293 cells were infected with AdgB-2 and labeled with [^{35}S]methionine, and gB was immunoprecipitated from cell extracts with anti-gB serum (Fig. 4a). With this vector, gB was expressed in both 293 and HeLa cells by 24 hr postinfection. The level of expression of gB in 293 cells was higher than in HeLa cells at this relatively early time, most probably because the replication of the virus progressed more rapidly in 293 cells. In subsequent experiments we compared the levels of gB synthesis in AdgB-2-infected human R970, mouse LTA, or Z4 cells (Persson *et al.*, 1985) to levels produced by corresponding HSV-1 infections (Fig. 4b). AdgB-2 induced readily detectable amounts of gB in R970 cells infected with 5 PFU/cell and even higher levels of gB when 50 PFU/cell was used. In all of the human cell lines infected with AdgB-2, polyclonal anti-gB serum precipitated a prominant band of approximately 50 Da as well as less prominant bands which are most probably derived by limited proteolysis of gB during extraction (Zezulak and Spear, 1984). In addition, though mouse LTA and Z4 cells both expressed gB after infection with AdgB-2, levels of expression in these mouse cell lines were much lower than in human cells and extensive morphological changes in mouse cells were not observed. We also noticed that the ratio of gB to pgB was greater in AdgB-2 than in HSV-infected L cells. Similar results have been observed with L cell lines transfected with

the HSV-2 gD gene (Johnson and Smiley, 1985) and the HSV-1 gB gene (Rosenthal *et al.*, 1987) versus cells infected with HSV-1.

Kinetics of gB expression in AdgB-1- and AdgB-2-infected cells

To compare the kinetics and levels of synthesis of gB during infections with AdgB-1, AdgB-2, and HSV-1, R970 cells were pulse-labeled with [^{35}S]methionine at various times after infection. The time course of expression of gB in HSV-1-infected cells followed the expected kinetics: gB was not detected at 3 hr postinfection; synthesis (detected as pgB) peaked at about 10 hr but continued until after 20 hr postinfection (Fig. 5). A small amount of gB was expressed in AdgB-1-infected cells but only after 60 hr consistent with the results obtained previously (Fig. 2). The replication of E1-deficient mutants of adenovirus on human cells is very slow (Nevins, 1981) but sufficient to account for the low level of expression of gB at late times in AdgB-1-infected cells. In contrast, the synthetic rate of gB in AdgB-2-infected cells was equal to or greater than that in HSV-1-infected cells. Synthesis of gB in AdgB-2-infected R970 cells did not peak until approximately 39 hr postinfection and did not drop dramatically late in the infection, even after 60–72 hr postinfection when cells began lifting off the dishes. Analysis of steady-state levels of gB in AdgB-2- versus HSV-1-infected cells using Western blotting indicated that there was approximately as much gB in AdgB-2-infected cells at 43 hr postinfection as in HSV-1-infected cells at 20 hr postinfection (results not shown).

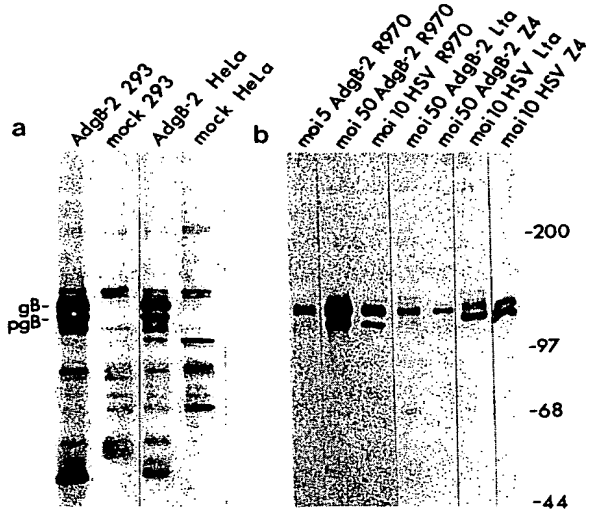


FIG. 4. Expression of gB in human 293, HeLa, or R970 cells and mouse LTA or Z4 cells infected with AdgB-2 or HSV-1. (a) 293 cells or HeLa cells were left uninfected (mock) or were infected with AdgB-2 (10 PFU/cell). After 20 hr the monolayers were washed twice with medium lacking methionine and labeled with [^{35}S]methionine. Four hours later cell extracts were made and gB was immunoprecipitated with rabbit anti-gB polyclonal serum No. 67. (b) Human R970 cells and mouse LTA and Z4 cells were infected with AdgB-2 (5 or 50 PFU/cell) or HSV-1 (10 PFU/cell). At 28 hr postinfection (AdgB-2) or 4 hr postinfection (HSV-1) the cell monolayers were washed twice with medium lacking methionine and labeled with [^{35}S]methionine for 8 hr. Cell extracts were made and gB was immunoprecipitated with monoclonal antibody 15βB2. Precipitated proteins were electrophoresed on polyacrylamide gels. The exposure of lanes involving mouse LTA or Z4 cells was four times longer than for R970 cells in (b). The postions of the mature (gB) and immature (pgB) forms of the glycoprotein are indicated on the left. Molecular weight markers of 200, 97, 68 and 44 kDa are indicated on the right.

Analysis of AdgB2 gB-related transcripts

The rationale for using the chimeric gB construct from pSV2gB in which gB coding sequences were linked to the SV40 early promoter was that gB expression might be driven by the SV40 promoter when the chimeric gene was inserted into an Ad5 genome. However, fragments of the SV40 large T antigen are transcribed from the E3 and major late promoters of Ad2 in Ad2–SV40 hybrid viruses (reviewed in Klessig, 1984), and a hepatitis B surface antigen gene inserted into the E3 region is driven from upstream Ad promoters in recombinant viral strains (Morin *et al.*, 1987). Thus, it was possible that gB was expressed in a similar fashion in the AdgB2 vector which carries the gB coding sequences in the same orientation as the major late and E3 promoters. To determine whether the SV40 promoter was utilized in AdgB2 infections, gB-related transcripts were studied by S1 nuclease mapping and primer extension.

The S1 probe and primer used in these experiments share a common 5′-end, and were therefore predicted to generate comigrating products of ca. 140 nt if the SV40 promoter element is used (Fig. 6). We were unable to detect such transcripts using RNA from AdgB-2-infected cells. Instead, AdgB-2 RNA gave rise to two protected fragments of 189 and 155 nt in S1 nuclease mapping experiments (lane 1). The 189-nt signal corresponds to fully protected probe, indicating that many gB-related transcripts initiated upstream of the SV40 promoter element. The 155-nt signal maps just downstream of the SV40 TATA box, at a site upstream of the predicted initiation site of SV40 promoter-driven mRNAs. Primer extension analysis of AdgB-2 RNA generated a heterogeneous family of products (lanes 3 and 4), all of which were considerably larger than that predicted for RNAs initiated from the SV40 promoter element (140 nt). Because we were unable to detect primer extension products corresponding to the S1 signal at ca. 155 nt, we conclude

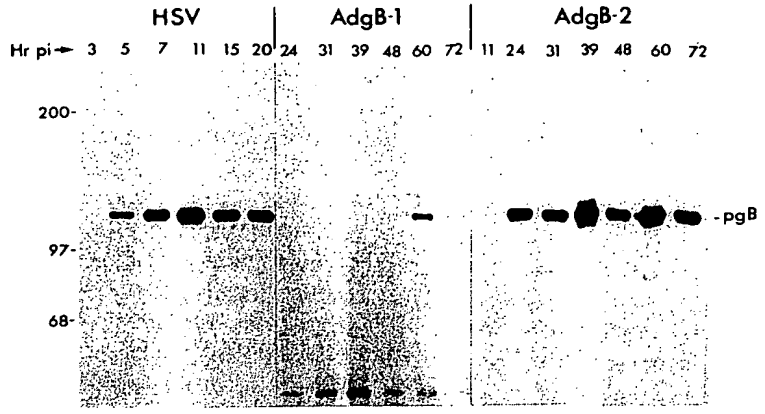


FIG. 5. Time course of gB expression in HSV-1-, AdgB-1-, and AdgB-2-infected cells. R970 cell monolayers were infected with HSV-1 (10 PFU/cell), AdgB-1 (20 PFU/cell), or AdgB-2 (20 PFU/cell) and after various times (hr p.i.) the monolayers were washed three times with medium lacking methionine and pulse-labeled with [^{35}S]methionine for 10 min. Cell extracts were made and gB was immunoprecipitated with monoclonal antibody 15βB2. The lanes showing gB synthesis in AdgB-1-infected cells were exposed to film six times longer than those for HSV- and AgB-2-infected cells. The position of pgB, the immature form of gB, is shown at right and positions of molecular weight markers of 200, 97, and 68 kDa are shown at left.

that this S1 signal is most likely derived from one or more spliced RNAs initiated upstream of the SV40 promoter element. Control experiments in which mRNA prepared from HSV-1-infected cells was used gave the expected results: an S1 product of ca. 74 nt corresponding to the break in complementarity between HSV-1 gB mRNA and the probe derived from the SV40–gB chimeric gene, and a primer extension product of 110 nt, corresponding to the known 5′-end of HSV-1 gB transcripts. Thus, the results shown in Fig. 6 indicate that the SV40 promoter was not being utilized to any significant extent in AdgB-2-infected cells, and suggest that expression of gB is due to promoters mapping to the left of the gB–SV40 gene (such as the E3 or major late promoter). The precise structure of gB-encoding transcripts in AdgB-2 is a subject for further study.

Subcellular localization of gB in AdgB-2-infected cells

HSV glycoproteins are processed and transported to the surfaces of cells transfected with glycoprotein genes (Arsenakis *et al.*, 1986; Johnson and Smiley, 1985), although there is evidence that this transport is more rapid in the absence of HSV infection (Johnson and Smiley, 1985). To examine the subcellular distribution of gB in AdgB-2- and HSV-1-infected cells, the cells were fixed, in some cases permeabilized with nonionic detergent, and stained with a mouse monoclonal antibody and fluoresceinated rabbit anti-mouse antibodies (see Fig. 7A–D). Cells infected with AdgB-2, and not permeabilized (C), displayed a stippled, patchy type of surface fluorescence similar to that found on the surface of HSV-infected cells (A) suggesting that gB was aggregated into microdomains of the surface of both HSV- and AdgB-2-infected cells. When HSV-1-infected cells were permeabilized (B), anti-gB antibody stained the nuclear envelope and various intracellular membraneous structures as well as the cell surface. Nuclear envelope staining was particularly noticeable because nuclei swell after infection with HSV. In contrast, most of the intracellular gB in AdgB-2-infected cells (D) was contained in large cytoplasmic vacuoles although staining of the nuclear envelope and cytoplasmic membranes was also observed.

Extensive vacuolization in AdgB-2-infected cells was also apparent by bright field microscopy (Fig. 7F) and resulted in a cytopathic effect which was strikingly different from that seen in wt Ad5-infected cells (Fig. 7E). It appeared that vacuoles observed using bright field microscopy correspond to vacules containing concentrated gB identified by fluorescent antibody staining.

Processing and intracellular transport of gB in AdgB-2- and HSV-infected cells

A number of studies have shown that pgB contains only high-mannose oligosaccharides and is principally localized to the nuclear envelope and rough endoplasmic reticulum whereas mature gB has complex high-mannose oligosaccharides and is principally found in the Golgi apparatus and on the cell surface (Compton and Courtney, 1984; Johnson and Spear, 1982; Wenske *et al.*, 1982). In both AdgB-2- and HSV-1-in-

fected cells mature gB was first detected after a 1-hr chase period (Fig. 8a) but a much larger fraction of pgB was converted to gB in AdgB-2-infected cells after a 3.5-hr chase period than was converted in HSV-1-infected cells. The conversion of pgB to gB was probably more extensive than depicted here because some incorporation of [^{35}S]methionine occurred during the chase periods in this particular experiment. The more efficient conversion of pgB to gB in AdgB-2-infected cells versus HSV-1-infected cells was not surprising since cell lines expressing HSV glycoproteins also process these polypeptides more efficiently and often more rapidly than HSV-1-infected cells (Johnson and Smiley, 1985; Rosenthal *et al.*, 1987).

To examine the kinetics with which newly synthesized gB reached the surface of AdgB-2-infected cells, cells were pulse-labeled with [^{35}S]methionine, label was chased for various times, and the cells were treated with trypsin to remove cell surface proteins (Fig. 8b). Approximately half of the pgB was converted to gB after a 1-hr chase period and a considerable fraction of the mature form of the protein was sensitive to trypsin (compare lanes C1− and C1+). After 2- or 3.5-hr chase periods (C2, C3) a larger fraction of the total gB was in the mature form and much of this was sensitive to trypsin. A large fraction of pulse-labeled gB is also sensitive to trypsin after 3.5 hr in HSV-1-infected cells (results not shown). Therefore, the bulk of the gB synthesized in AdgB-2-infected cells was transported, at least initially, to the cell surface although a significant fraction of the mature gB remained insensitive to exogenous protease. The trypsin-resistant fraction most probably remains in the cytoplasm of AdgB-2-infected cells but may also be trypsin-resistant for some other reason such as aggregation of gB.

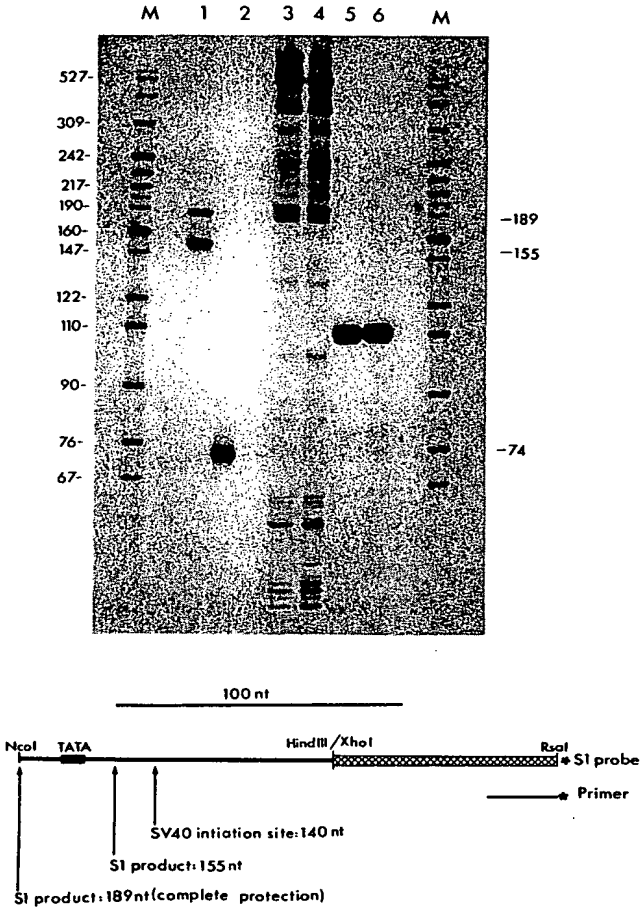
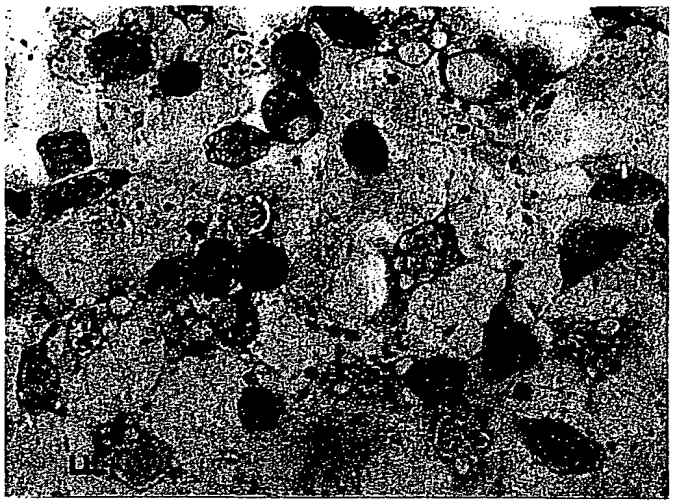
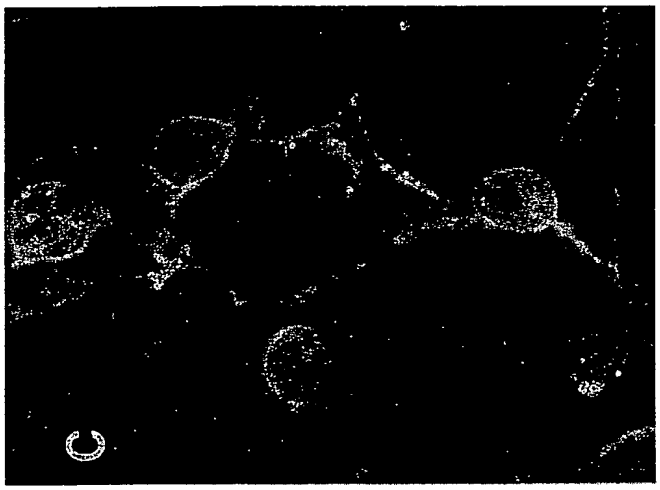


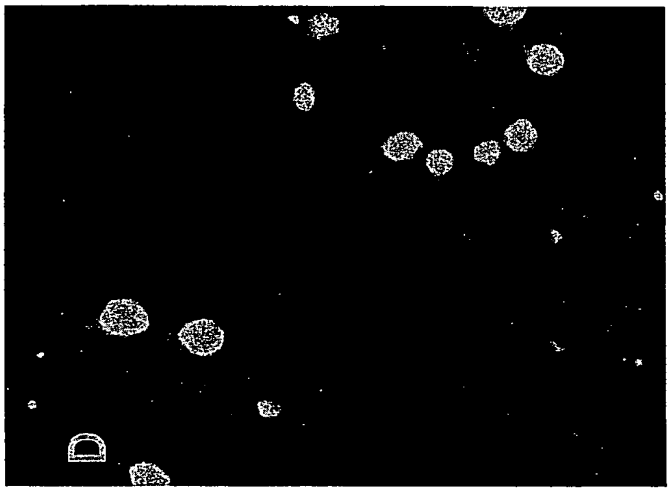
FIG. 6. S1 and primer extension analysis of AdgB2 gB-related transcripts. Total cytoplasmic RNA (20 μg) prepared from R970 cells 6 hr postinfection with HSV-1 (F) or 36 hr postinfection with AdgB-2 was hybridized to the indicated probe, and then treated with either S1 nuclease or AMV reverse transcriptase at 37 or 43°, as described under Materials and Methods. Products were displayed on an 8% sequencing gel. Lane 1, S1 analysis of AdgB-2 RNA; lane 2, S1 HSV-1 RNA; lane 3, primer extension, AdgB-2 RNA (43°); lane 4, primer extension, AdgB-2 RNA (37°); lane 5, primer extension, HSV-1 RNA (43°); lane 6, primer extension, HSV-1 RNA (37°). Markers (M) were 3′-labeled *Hpa*II fragments of pBR322 DNA.

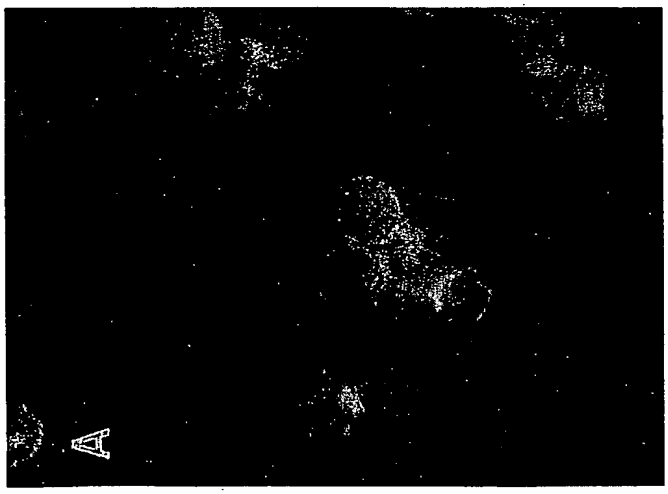
DISCUSSION

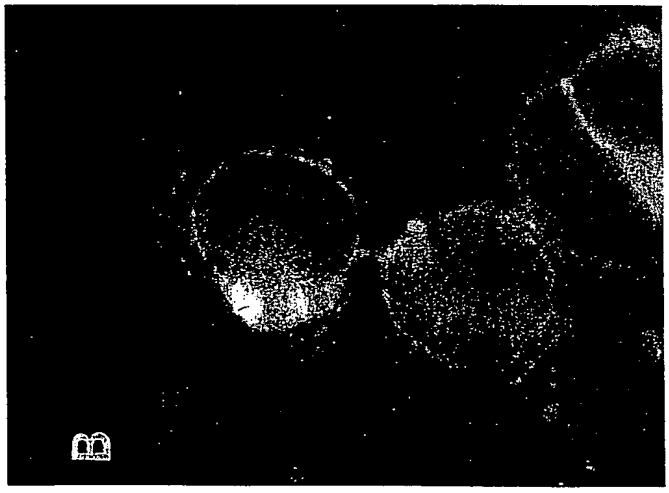
Adenovirus vectors have been used for the expression of a number of biologically important proteins and RNAs including polyomavirus middle T antigen (Berkner *et al.*, 1987; Davidson and Hassell, 1987), SV40 large T antigen (Gluzman *et al.*, 1982; Thummel *et al.*, 1981), HSV thymidine kinase (Haj-Ahmad and Graham, 1986), human globin RNA (Karlsson *et al.*, 1986), murine μ-immunoglobin RNA (Ruether *et al.*, 1986), and dihydrofolate reductase (Berkner *et al.*, 1987; Berkner and Sharp, 1984). In most of these examples, foreign genes were substituted for the E1 region of adenovirus, limiting replication of the vectors to cells capable of expressing E1 such as 293 cells (Graham *et al.*, 1977). Using infectious bacterial plasmids described previously (Ghosh-Choudhury *et al.*, 1986, 1987), we constructed two adenovirus vectors with substitutions in the E3 region of the HSV-1 gB gene coupled to the SV40 early promoter. AdgB-1, which lacks E1 sequences and therefore must be propagated in 293 cells, stimulated the expression of high levels of gB in 293 cells but very low levels of the polypeptide in other human or mouse cells and these were detectable only late in infection. In contrast, AdgB-2 having an intact E1 region, was able to replicate well in all the human cell lines tested and stimulated the synthesis of high levels of gB in human cells and to a lesser but still substantial degree in mouse cell lines. Expression of gB occurred throughout most











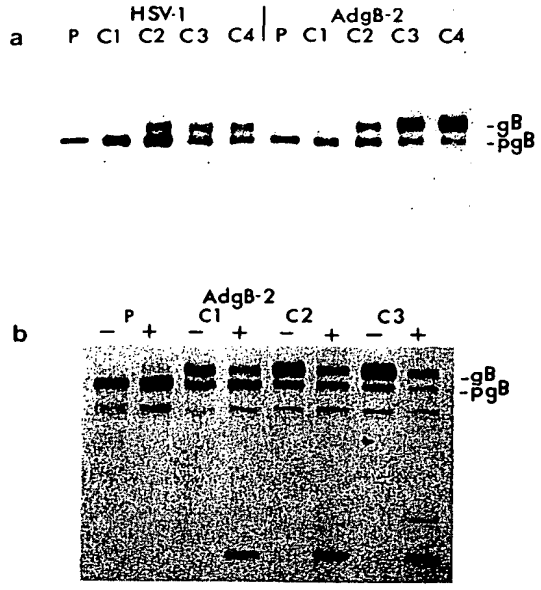


FIG. 8. Processing and transport of gB to the cell surface. (a) R970 cells were infected with HSV-1 (10 PFU/cell) or AdgB-2 (20 PFU/cell) and labeled at 7 hr postinfection (HSV-1) or 38 hr postinfection (AdgB-2) with [^{35}S]methionine for 10 min or labeled for 10 min and incubated with medium containing excess unlabeled methionine for 30 min (C1), 60 min (C2), 120 min (C3), or 210 min (C4). Cell extracts were made and gB was immunoprecipitated with monoclonal antibody 15βB2. (b) Cells infected with AdgB-2 were labeled as described above except that the chase periods were 60 min (C1), 120 min (C2), and 210 min (C3). Cell monolayers were then washed with PBS, and incubated with PBS(−) or PBS containing trypsin (+) for 10 min at 37°. the trypsin was inactivated with PMSF, cell extracts were made, and gB was immunoprecipitated with monoclonal antibody 15βB2. The positions of gB and the immature form pgB are indicated.

of the infectious cycle of AdgB-2 although synthesis of gB was not detected early, i.e., prior to 10 hr postinfection.

The lack of expression of gB early after infection of HeLa cells with AdgB-1 was somewhat surprising in light of our previous observations that gB synthesis could be easily detected in human or monkey cells transfected with pSV2gB (D. Johnson, unpublished). Reasoning that the adenovirus vector would deliver transcriptionally active copies of the SV2gB hybrid gene into these cells, we expected to be able to detect gB early in the infection especially when high multiplicities of infection were used. Since this was not the case, the expression of gB may require replication of the adenovirus vector DNA. Consistent with this hypothesis was the observation that synthesis of gB was particularly low in mouse cells where replication of AdgB-2 is poor.

Although the gB coding sequence present in AdgB-2 was linked to the SV40 early promoter, we were unable to detect gB-related transcripts initiated from the SV40 promoter. Instead, all detectable gB-related RNAs appeared to initiate upstream of the SV40 promoter. From the size and heterogeneity of cDNA species produced by primer extension it seems certain that at least some of the transcripts must initiate in upstream Ad5 sequences, and possibly involve the use of more than a single promoter and a variety of different splicing events. A subset of these transcripts appeared to splice to a site just downstream of the SV40 TATA box and it seem likely that the sequence CCGAGG present at this position in SV40 served as a cryptic splice acceptor. It is not yet clear which of the gB-related transcripts that we have detected are translated to give rise to the gB polypeptide expressed in AdgB-2-infected cells. Nevertheless, our results indicate that a functionally promoterless coding sequence inserted into the E3 region of Ad5 can give rise to high levels of translatable mRNA. However, the fact that AdgB-1 gave rise to high levels of gB protein in infected 293 cells indicates that expression is not restricted to genes inserted in the left to right orientation. We have also constructed an E1 containing adenovirus vector analagous to AdgB-2 which has the gB–SV40 chimeric gene in the reversed, i.e., right to left orientation. Human R970 cells infected with this vector expressed gB although at lower levels than in AdgB-2-infected cells. Since adenovirus E1A gene products have been reported to repress SV40 enhancer activity (Borrelli *et al.*, 1984; Velcich and Ziff, 1985) we cannot say whether this vector and AdgB-1 utilize the SV40 promoter for expression or rely on transcription originating to the right, for example in region E4.

One notable observation to come out of these studies was that sustained, high-level expression of gB markedly affected the cell morphology: cells infected with AdgB-2 rapidly became extensively vacuolated in contrast to cells infected with Sub54 virus which is virtually indentical to AdgB-2 except for the absence of the SV2gB gene. The vacuoles which accumulated in the cytoplasm of AdgB-2-infected cells appeared to

FIG. 7. Distribution of gB in HSV-1- and AdgB-2-infected cells. (A–D) R970 cells growing on glass coverslips were infected with HSV-1 or AdgB-2 and fixed with 4% formaldehyde at 15 hr (HSV-1) or 44 hr (AdgB-2) postinfection. Fixed cells were either permeabilized with 0.1% Triton X-100 or left nonpermeabilized prior to staining with anti-gB monoclonal antibody 15βB2 and fluoresceinated rabbit anti-mouse IgG. HSV-infected, nonpermeabilized or permeabilized cells are shown in (A) and (B), respectively, and AdgB-2-infected nonpermeabilized or permeabilized cells are shown in (C) and (D). (E) and (F), respectively, show results of bright field microscopy of cells infected with Sub54 or AdgB-2 (37 hr postinfection).

contain high concentrations of gB, and may have been induced by the expression of gB. It is noteworthy that in a recent study in which high-level expression of the lymphocytic choriomeningitis virus glycoproteins was attained using baculovirus vectors, extensive vacuolization of infected cells was also observed (Matsuura *et al.*, 1987). Thus vacuolization associated with high-level viral glycoprotein synthesis in two such different systems may suggest this is a general or at least common phenomenon related to over expression of membrane glycoproteins.

The distribution of gB on the surfaces of cells infected with AdgB-2 was extremely nonuniform, with the glycoprotein largely aggregated, as is also observed late in HSV-1 infections. Oligomers of gB have been detected in extracts of virions (Sarmiento *et al.*, 1979) and infected cells (Claesson-Welsh and Spear, 1986; Haffey and Spear, 1980). Therefore, if gB oligomerizes or aggregates on the surfaces of AdgB-2-infected cells it may also be endocytosed producing vacuoles or endosomes which contain concentrated gB. In support of this hypothesis, we found that a large fraction of the gB synthesized in AdgB-2-infected cells is transported at least initially to the cell surface when much of the steady-state gB is localized to vacuoles. It is also possible that high-level expression of gB may induce vacuolation of cells by other mechanisms. The predicted structure of gB suggests that the polypeptide may traverse the lipid bilayer three times and it has been suggested that this structure might act as an ion channel (Pellett *et al.*, 1985b) which could consequently perturb intracellular ionic concentrations after HSV infection (Fritz and Nahmias, 1972). Thus, sustained high-level expression of gB may also lead to accumulation of high concentrations of certain ions and cause vacuolation. Ionophores such as monensin also cause extensive vacuolation of cells (Tartakoff and Vassalli, 1977) and accumulation of viral glycoproteins in the vacuoles (Johnson and Schlesinger, 1980; Johnson and Spear, 1982).

Our finding that gB is largely concentrated in cytoplasmic vacuoles in cells infected with AdgB-2 and not in HSV-1-infected cells may be explained by two differences between the two types of virus-infected cells. First, cells infected with AdgB-2 accumulate gB over a much longer period than HSV-1-infected cells. Second, in HSV-infected cells, viral glycoproteins are packaged into virion envelopes in the nuclear envelope and then transported via the Golgi apparatus to the cell surface where a large fraction of virions remain bound to the cell surface (Johnson and Spear, 1982; Spear, 1984). This process cannot occur in AdgB-2-infected cells and thus gB most probably accumulates on the cell surface and may enter cytoplasmic vacuoles by endocytosis.

Increasing attention has been focussed on the use of recombinant viruses as potential vaccines. The use of vaccinia virus vectors is well advanced and their efficacy in animal immunization has been demonstrated (Kieny, *et al.*, 1984; Smith *et al.*, 1983). However, toxic effects of the virus (Jones *et al.*, 1986) may limit their widespread acceptance. Herpesviruses may also be suitable for some specific applications (Roizman and Jenkins, 1985) but they have the disadvantages of being pathogenic and difficult to manipulate. Adenovirus serotypes 4 and 7 have been extensively used in vaccination against acute respiratory disease and have been administered to large numbers of military recruits, and found to be safe and effective (Dudding *et al.*, 1972; Meiklejohn, 1983; Takafuji *et al.*, 1979). In addition, adenoviruses are relatively stable and grow to very high titers, and the vaccines can be administered orally. They replicate in the upper respiratory and gastrointestinal tracts of man and a number of vertebrates and thus may be advantageous for vaccination against viruses which replicate at or near these sites such as influenza viruses, respiratory syncytial viruses, or perhaps HSV. We are presently testing the protective value of AdgB-2 vaccination in mice and monkeys and preliminary results have shown that anti-gB antibodies are produced in mice infected with AdgB-2. Because adenovirus vectors can infect a wide spectrum of mouse and human cells, including macrophages, vectors such as those described here will undoubtedly be useful in studies of the specificity of anti-HSV cytotoxic T lymphocytes (CTL) as well as in other studies where efficient expression of antigens is needed. Preliminary results suggest that mouse cells infected with AdgB-2 are not lysed by anti-HSV CTL which supports our previous finding that gB is not a CTL target in mice (Rosenthal *et al.*, 1987). We are attempting to use adenovirus vectors to express HSV-1 gC, which acts as a murine anti-HSV CTL target, so that we can extend the CTL studies to humans.

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